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Progress in Retinal and Eye Research 24 (2005) 355–377

Progress in
RETINAL AND EYE RESEARCH

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Nonproliferative retinopathy in diabetes type 2. Initial stages and characterization of phenotypes

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Abstract

This review addresses the initial stages of nonproliferative diabetic retinopathy in diabetes type 2.

The natural history of the initial lesions occurring in the diabetic retina has particular relevance for our understanding and management of diabetic retinal disease, one of the major causes of vision loss in the western world. Diabetic retinal lesions are still reversible at this stage opening entirely new opportunities for effective intervention.

Four main alterations characterize these early stages of diabetic retinopathy: microaneurysms/hemorrhages, alteration of the blood–retinal barrier, capillary closure and alterations in the neuronal and glial cells of the retina. These alterations may be monitored by red-dot counting on eye fundus images and by fluorescein leakage and retinal thickness measurements.

A combination of these methods through multimodal macula mapping has contributed by identifying three different phenotypes of diabetic retinopathy. They show different types and rates of progression which suggest the involvement of different susceptibility genes. The identification of different phenotypes opens the door for genotype characterization, different management strategies targeted treatments.

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doi:10.1016/j.preteyeres.2004.07.004

Diabetic retinopathy is a chronic retinal disorder that eventually develops, to some degree, in nearly all patients with diabetes mellitus. Diabetic retinopathy is characterized by gradually progressive alterations in the retinal microvasculature and is the leading cause of new cases of legal blindness among Americans between the ages of 20 and 74 years of age (Aiello et al., 1998).

Diabetic retinopathy occurs in both type 1 (also known as juvenile-onset or insulin-dependent diabetes) and type 2 (also known as adult-onset or noninsulin-dependent diabetes) diabetes. All the features of diabetic retinopathy may be found in both types of diabetes but characteristically the incidence of the main causes of vision loss, macular edema and retinal neovascularization, is quite different for each type of diabetes (Aiello et al., 1998). Diabetic retinopathy in type 1 diabetes induces vision loss mainly due to the formation of new vessels in the eye fundus and development of proliferative retinopathy, whereas in type 2 diabetes vision loss is most commonly due to macular edema and proliferative retinopathy is relatively rare.

It is apparent, from the data available from a variety of large longitudinal studies, that the evolution and progression of diabetic retinopathy vary according to the types of diabetes involved, showing dissimilarities among different patients even when belonging to the same type of diabetes and does not necessarily progress in every patient to proliferative retinopathy. We will address in this review only the initial stages of nonproliferative retinopathy in diabetes type 2.

1. Present classifications of nonproliferative diabetic retinopathy

There have been many attempts to classify the lesions observed in the retina in diabetes. The first such classification that resulted from consensus and was accepted internationally was the Airlie House classification.

The Airlie House Classification was developed by a 12-member committee during a Symposium on the Treatment of Diabetic Retinopathy held at Airlie House in Warrington, VA, in September 1968 (Goldberg and Fine, 1969). The goal of its authors was to provide a simple scheme for expressing the presence and severity of the fundus lesions commonly seen in this disorder that were suitable for ophthalmoscopy or fundus photography.

Like most other classifications of diabetic retinopathy, the early treatment diabetic retinopathy study (ETDRS) (Early Treatment Diabetic Retinopathy Study Research Group, 1991) modification of the Airlie House classification is limited to assessing the severity and/or extent of various characteristic abnormalities but does not provide an overall severity scale and has been tested solely for evaluation of progression to proliferative diabetic retinopathy.

In the diabetic retinopathy study (DRS) (Diabetic Retinopathy Study Research Group, 1981), a useful definition was proposed for only one part of the picture, the severe stage of nonproliferative diabetic retinopathy (NPDR). In this definition, which was based on clinical impression and quantitative clinical descriptions, four abnormalities were considered: hemorrhages and/or microaneurysms, cotton-wool spots (soft exudates), intraretinal microvascular abnormalities (IRMA's) and venous beading. Additional DRS analyses demonstrated that hemorrhages and/or microaneurysms and venous beading were the more powerful predictors of visual loss.

The ETDRS adopted the DRS definitions of severe NPDR and contributed by defining moderate NPDR in a more detailed scale. It is interesting that these scales and classifications were all based in a variety of complex statistical analyses all focused on the percentage of eyes with progression to proliferative diabetic retinopathy. The ETDRS-based classification of severity and progression of diabetic retinopathy is entirely based on the assumption that the retinopathy is homogeneous and will ultimately progress to proliferative retinopathy. Detailed information on the segment of the final scale dealing with mild-to-severe NPDR is related to 1-, 3- and 5-year rates of proliferative diabetic retinopathy.

The ETDRS research group in their Report number 12 clearly state that "because few eyes were included in the ETDRS that had very mild retinopathy the definitions of the final scale regarding the lower range of NPDR could not be examined" (Early Treatment Diabetic Retinopathy Study Research Group, 1991). For example, it could not determine whether presence of mild retinal hemorrhages in addition to microaneurysms was sufficiently less severe than the presence of hard or soft exudates to merit designation of separate levels for these two groups (i.e., division of level 35 into two parts).

The available classifications of diabetic retinopathy give very useful information but have two major problems.

One, is the lack of information on the mild stage of the disease, which is clearly the most interesting stage because it is the one that shows some degree of reversibility and may respond to improved medical treatment and new forms of treatment.

The second, is the fact that the ETDRS classification, which is considered the reference classification, was constructed on the basis that diabetic retinopathy, given time, uniformly progresses to proliferative retinopathy. It assumes that retinal neovascularization and proliferative retinopathy are direct consequences of diabetes. There are, on the contrary, many arguments to support the thesis that proliferative retinopathy occurs in diabetes as a result of the extensive capillary closure and ischemia and is, in itself, relatively independent of the diabetic general metabolic status (Cunha-Vaz, 1978).

Other classifications have been proposed recently in an effort to simplify the ETDRS final scale (Wilkinson et al., 2003). Their goal has been to establish references for clinical studies and to facilitate clinical follow-up of diabetic retinopathy. They do not address the need for more detailed characterization of initial stages of diabetic retinal disease and therefore are not discussed in this review.

2. Natural history of the initial stages of NPDR

The fundus abnormalities that are identified on clinical examination of mild to moderate NPDR include microaneurysms and/or hemorhages, i.e., red-dots in the fundus, and exudates.

The initial stages of NPDR are, therefore, characterized by the presence of red-dots (microaneurysms and/or hemorhages) and indirect signs of vascular hypermeability and capillary closure, i.e., both hard and soft exudates or cotton-wool spots, respectively.

These are the alterations that dominate the initial stages of NPDR and we will analyze their development and progression, in order to clarify their relative importance in the progression of diabetic retinopathy. They are not present in every patient in the same way nor at the same rate.

It must be realized that the course and rates of progression of the retinopathy vary between patients. Microaneurysms, for example, may come and go. Once you get a microaneurysm you do not necessarily continue to have that microaneurysm. Microaneurysms may disappear due to vessel closure, which is an indication of worsening of the retinopathy because of progressive vascular closure (Cunha-Vaz, 1992). Hemorrhages will obviously come and go as the body heals them. Clinical improvement may be apparent but in reality it masks the worsening of the disease.

A prominent feature of diabetic retinopathy, diabetic macular edema, can spontaneously resolve itself. Indeed, it is resolved in approximately a third of patients over a period of 6 months, without any intervention. (Ferris and Davis, 1990)

The initial pathological changes occurring in the diabetic retina are characteristically located in the small retinal vessels of the posterior pole of the retina, that is, in the macular area. The structural changes in the small vessels include endothelial cell and pericyte damage and thickening of basement membrane (Cunha-Vaz, 1978; Garner, 1987).

Normally, retinal vascular endothelium is a fundamental part of the blood–retinal barrier (BRB), which has many similarities with the blood–brain barrier. It functions as a selective barrier which has shown to be altered in experimental and human diabetes (Cunha-Vaz et al., 1975).

Pericyte damage has been reported as one of the earliest findings in diabetic retinal disease since the introduction of retinal digest studies (Cogan and Kwabara, 1963). However, pericyte apoptosis is more readily detectable than endothelial cell apoptosis, most probably because the pericytes are encased in basement membrane and thus, less accessible to clearing mechanisms, whereas apoptotic endothelial cells slough off into the capillary lumen and are cleared by blood flow.

The simplest paradigm that explains capillary permeability and closure centers on the vascular endothelium. In the retina, endothelial cells are the site of the BRB, a specific blood-tissue barrier, and, as in all vessels, provide a nonthrombogenic surface for blood flow. Both these properties are eventually compromised by diabetes.

On the other hand, diabetes also affects the neural and glial cells of the retina. Consequently, we have an initial pathological picture characterized by endothelial and pericyte alterations associated with basement membrane thickening and microaneurysm formation. These alterations are characteristic for the retina, particularly the alteration of the BRB, the pericyte damage and the microaneurysm formation, but occur in a variety of diseases unrelated to diabetes. There is clear site specificity, not disease specificity (Cunha-Vaz, 1978).

Which are then the features of the retinal circulation which are specific to the retina and may be responsible for the site specificity of diabetic retinopathy? They are the BRB and the autoregulation of retinal blood flow. Both serve the needs of the neuronal and glial cells of the retina.

An abnormality of the BRB, demonstrated both by vitreous fluorometry and fluorescein angiography is an early finding both in human and experimental diabetes (Cunha-Vaz et al., 1975; Waltman et al., 1978a, b).

Fluorescein leakage is one of the earliest findings in diabetic retinal disease, demonstrating the alteration of

the BRB. It appears to lead directly to macular edema, which remains the most frequent cause of visual loss in diabetes.

Another important characteristic of the retinal circulation is its capacity to autoregulate and compensate variations in blood pressure, ocular tension, etc., maintaining a relatively uniform blood flow. Changes in retinal blood flow have, indeed, been reported in both human and experimental diabetes (Kohner, 1977).

Capillary closure leading to retinal ischemia and inducing neovascularization and proliferative retinopathy, leads to the most tragic outcomes for visual loss: vitreous hemorrhage, rubeosis iridis, retinal detachment, etc. It is becoming apparent that at least three processes can contribute to retinal capillary occlusion and obliteration in diabetes: proinflammatory changes, microthrombosis, and apoptosis (Gardner and Aiello, 2000). These processes have been documented in both human and experimental diabetes. There are indications taken from experimental studies that proinflammatory changes and leukostasis are early events and that microthrombosis and apoptosis occur subsequently.

2.1. Microaneurysms/hemorrhages. Red-dots formation and disappearance rates

Microaneurysms (MA) and hemorrhages (HEM) identified as red-dots are the initial changes seen on ophthalmoscopic examination and fundus photography (SFP). They may be counted and red-dot counting has been suggested as an appropriate marker of retinopathy progression (Klein et al., 1995a, b).

It must be realized that red-dot formation and disappearance are dynamic processes. During a 2-year follow-up of 24 type 1 diabetics with mild background diabetic retinopathy using fluorescein angiography, Hellstedt and Immonen (1996) observed 395 new MA and the disappearance of 258 previously identified.

Generally, the disappearance of a MA is not a reversible process and indicates vessel closure and progressive vascular damage. Therefore, to assess progression of retinopathy, red-dot counting should take into account every newly developed red-dot identified in a new location.

We have developed software for assisted red-dot counting in fundus-digitized images where the location of each red-dot is taken into account and registered (Fig. 1). In this way, in a follow-up study with repeated fundus images obtained at regular intervals, all red-dots in the fundus were counted and added as they became visible in new locations in the retina. The results of red-dot counting using this method, in a 2-year follow-up study of a series of eyes with mild nonproliferative retinopathy in subjects with type 2 diabetes maintaining a stable metabolic control during the period of the study suggest that red-dot counting may be a good marker of disease progression in the initial stages of NPDR (Torrent-Solans et al., 2004).

Fig. 1. Red-dot identification by location in the macula in a 2-year period of follow-up at the initial visit VS, 6 (V6), 12 (V12), 18 (V18) and 24 (V24) month.

Fifty eyes from 50 patients, with type 2 diabetes mellitus and mild nonproliferative retinopathy, were prospectively followed. These were consecutive patients that fulfilled the inclusion criteria. Treated with oral hypoglycemic agents, they maintained a stable metabolic control.

One selection criterion was the presence of at least one red-dot at the first visit in field 2 of the 7-field stereo fundus photography (SFP) assessed by two independent readers.

Fundus photographs were taken every 6 months. Field 2 (centered in the macula) was chosen for the analysis of the presence of red-dots since this is the most important area in terms of potential visual impairment.

In order to improve human grader reliability in the identification and counting of red-dots on color fundus images, the software included algorithms for eye movement compensation, color correction and identification of each red-dot by its coordinates.

Using the software's ability to identify each red-dot as a single entity, in a specific location with identifiable coordinates, the following parameters were assessed:

1. Cumulative number of red-dots. This number is achieved using the main advantage of the new software, its ability to consider each red-dot as a single entity, identified by its specific location. Therefore, a red-dot identified on a new location is recognized as a new red-dot and added.
2. Red-dot formation rate. Red-dot formation rate is the annual rate of change over the study period. It is computed dividing the difference of the cumulative number of red-dots between the last and first visits by the 2 years period.
3. Red-dot disappearance rate. Red-dot disappearance rate is the sum of the red-dots that disappeared

during the study period divided by the 2 years of follow-up. A disappeared red-dot is one missing red-dot from a previous visit that will not show up again (during the study period).

Using the traditional procedure, the total amount of red-dots detected at every visit remained stable. However, the cumulative number of red-dots raised from 115 at the first visit to 505 at the last visit, showing a marked increase in new red-dots. These figures emerged because of the software's potential for counting every red-dot as a single entity once it was identified by its specific location. It is now obvious that there were many more red new dots in the fundus, i.e., microaneurysms and small hemorrhages, in this 2-year time period than expected using data for each examination separately.

One of the advantages of the method used is the ability to count the number of real new red-dots appearing at every visit (red-dot formation rate). The rate of formation (red-dots/year) ranged from 0 to 22. The results showed that eyes in the same retinopathy stage from different patients show very different red-dot formation rates. Values for red-dot formation rate higher than 3/year correlated well with increased fluorescein leakage measured by vitreous fluorometry ($p<0.001$) and capillary closure identified by a damaged foveal avascular zone (FAZ) ($p<0.038$), demonstrating a direct correlation with faster retinopathy progression.

The rate of disappearance (red-dots per year) ranged from 0 to 16. Red-dot disappearance rates also varied quite markedly in eyes from different patients and showed similar correlations.

In a recently developed International Clinical Diabetic Retinopathy Disease Severity Scale, developed under the auspices of the American Academy of Ophthalmology, the identification of microaneurysms and hemorrhages characterize the initial, mild stage, of diabetic retinopathy (Wilkinson et al., 2003).

Microaneurysms and hemorrhages identified by fundus photography as red-dots are considered the first clinical sign of retinopathy. Microaneurysm formation has been associated with localized proliferation of endothelial cells, loss of pericytes and alterations of the capillary basement membrane, alterations that occur in the initial stages of diabetic retinal disease and have been considered to be directly involved in its pathophysiology (Ashton, 1963, 1974; Cunha-Vaz, 1978).

Microaneurysm closure and disappearance is most probably due to thrombotic phenomena leading to subsequent rerouting of capillary blood flow and progressive remodeling of the retinal vasculature in diabetes (Boeri et al., 2001). These thrombotic changes are probably enhanced by changes in the red and white cells occurring as a result of diabetes. The presence and number of microaneurysms and their rates of formation and disappearance are, therefore, good candidates as markers of retinal vascular remodeling and may be good indicators of retinopathy progression.

Red-dot counting on fundus photographies and microaneurysm counting on fluorescein angiography have been proposed as predictive indicators for progression of diabetic retinopathy (Kohner et al., 1986). Our specially developed software allows the identification of the exact location of each red-dot in successive fundus photographs performed in each eye. Identification of the exact location of an individual red-dot is considered particularly important because a new microaneurysm is considered to develop only once in a specific location, its disappearance being generally associated with capillary closure, leaving in its place mainly remnants of basement membrane (Ashton, 1974; Cunha-Vaz, 1978).

Our study demonstrated a steady turnover of red-dots in the diabetic retina, even in the initial stages of retinopathy. In fact, most red-dots show a lifetime of less than 1 year, with new ones being formed and disappearing at rates which vary between different patients, confirming previous reports (Kohner and Dollery, 1970).

Most interestingly, however, is the observation that some patients show much higher rates of red-dot formation and disappearance, suggesting that they may represent a specific phenotype of diabetic retinopathy. These eyes showed faster retinopathy progression, with increased fluorescein leakage, i.e., alterations of BRB, and shorter duration of diabetes.

Red-dot counting on fundus photography instead of microaneurysm counting on fluorescein angiography is particularly promising because fundus photography is noninvasive and well accepted by the patients, particularly when involving repeated examinations.

In conclusion, our results, based on precise identification of the location of each red-dot on fundus photograph of diabetic eyes, suggest that red-dot formation and disappearance rates may be appropriate indicators of retinopathy progression, identifying in this simple way a diabetic retinopathy phenotype characterized by rapid retinopathy progression.

2.2. Alteration of the BRB. Fluorescein leakage measurements

Since the early 1950s, two research groups have contributed significantly to our understanding of the pathological picture of diabetic retinopathy: Ashton and co-workers in London (Ashton, 1963) and Cogan and his co-workers in Boston (Cogan and Kwabara, 1963).

From their observations the endothelial cells and the pericytes were seen to be affected from the earlier stages of diabetic retinopathy. When the BRB was found to be located primarily at the level of the endothelial membrane of retinal vessels (Shakib and Cunha-Vaz, 1966) it was only natural to assume that an alteration of

the endothelial cells could play a major role in diabetic retinal diseases. Ashton, in his 1965 Bowman Lecture, stated that early lesions of diabetic retinopathy are "focal breakdowns of the BRB" (Ashton, 1965).

The advent of fluorescein angiography confirmed most of what was known about the initial pathological picture of diabetic retinopathy and showed in the initial stages of the disease focal leaks of fluorescein, demonstrating, in a clinical setting, the existence of focal breakdowns of the BRB.

In 1975, vitreous fluorometry, a clinical quantitative method for the study of the BRB, was introduced by our group (Cunha-Vaz et al., 1975), showing that an alteration of the BRB could be detected and measured in some diabetic eyes with apparently normal fundi. These results were confirmed by Waltman et al. (1978b).

Thereafter, many experimental and clinical studies have examined the alteration of the BRB in diabetes with conflicting results at times, but showing in general that an alteration of the BRB is present in the diabetic retina and may have an important role in its development and progression (Cunha-Vaz, 2000a, b).

The damaged capillaries leak their contents intraretinally, resulting in the formation of hard yellow exudates (confluents of lipids and lipoproteins) in the nerve fiber layer and edema. Localized hemorrhages also result from the excessive vascular porosity. If present, hemorrhages take the form of dots and blots, an appearance attributable to their deep location and sequestration of blood in an anatomically compact retina.

Breakdown of the BRB plays, therefore, an important initiating role in the development of the pathological picture of diabetic retinopathy.

An alteration of the BRB has, indeed, been documented in a variety of studies using different models of experimental diabetes. These studies, initiated by Waltman and co-workers, (Waltman et al., 1978a) showed an alteration of the BRB in rats with streptozotocin-induced diabetes, well demonstrated by vitreous fluorometry, soon after the induction of chronic hyperglycemia. Futhermore, this alteration of the BRB was reversed by the administration of insulin and regularization of glycemia.

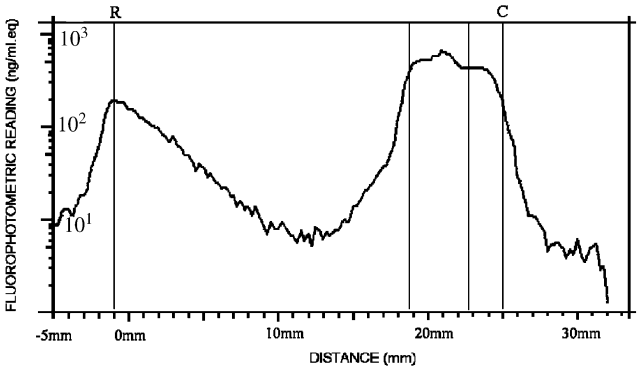

Fig. 2. Vitreous fluorophotometry recording from an eye with nonproliferative retinopathy from a patient with diabetes type 2 obtained 1 h after intravenous fluorescein injection. Note the fluorescence curve in the vitreous across an altered BRB. Reference landmarks: R-retina; C-cornea.

The alteration of the BRB in the rat with streptozotocin-induced diabetes occurs only 1 week after the administration of streptozotocin. There have been, however, contradicting reports regarding this site of the BRB breakdown. Studies using horseradish peroxidase as a tracer for electron microscopic investigation, pointed to the retinal pigmented epithelium as the main structure affected (Tso et al., 1980). However, studies using histochemical localization of naturally occurring albumin, performed by Murata et al. (Murata et al., 1993) and Viñores et al. (Viñores et al., 1990) have clearly shown that the main site of increased permeability of the BRB is located at the level of the inner BRB involving the retinal vessels. More recently, our group in Coimbra have demonstrated using confocal microscopy that the breakdown of the BRB occurring in rats 1 week after onset of streptozotocin-induced diabetes is localized preferentially in the inner BRB (Carmo et al., 1998).

In alloxan-induced diabetes, Engerman was able to demonstrate in the dog the development of a retinopathy presenting many of the features seen in man (Engerman, 1976). Ultrastructural studies, using horseradish peroxidase, a relatively large protein, demonstrated breakdown of the BRB in eyes showing signs of microvascular alterations. The breakdown of the inner BRB was manifested by the presence of the tracer in the cytoplasm of the endothelial cells and in ruptured junctions. It is noteworthy that the breakdown of the BRB was observed preferentially in vessels showing signs of endothelial proliferation.

Clinical studies on the application of vitreous fluorometry to diabetes were reported for the first time in 1975 (Cunha-Vaz et al., 1975). The examination of a series of predominantly adult-onset diabetics with apparently normal fundi revealed the frequent presence of an alteration of the BRB. The fluorescein concentration curves in the vitreous in the diabetic patients followed a gradient indicating penetration of fluorescein across the BRB (Fig. 2).

During the following years many research efforts were directed at improving the instrumentation and standardization of the method. An ocular fluorometer described by Zeimer et al. finally became available commercially, thus making it possible to repeat studies at different centers avoiding much of the variability in instrumentation that played some of the earlier studies (Zeimer et al., 1983).

An European multicenter study involving six different research groups showed that vitreous fluorometry,

performed using the Fluorotron Master and following a well-defined protocol, is a highly sensitive and reliable method for measuring the permeability of the BRB (Van Schaik et al., 1997).

Values for the BRB permeability coefficient obtained by different authors have shown good agreement. The European Community Network of Ocular Fluorometry found a mean value of $1.97 \pm 0.93 \times 10^{-7}$ cm/s on data collected from six centers for a total of 81 healthy volunteers.

A general review of the many studies performed using vitreous fluorometry, in both type 1 and 2 diabetes, shows that there is, in both types of diabetes, an alteration which is always present after development of ophthalmoscopically visible retinopathy and present in some eyes even before the development of clinically visible retinopathy (Cunha-Vaz et al., 1986). This breakdown of the BRB increases with duration of the disease and is associated with poor metabolic control.

We have followed in a 7-year prospective follow-up study a group of 40 patients with adult-onset diabetes mellitus, with retinopathy no greater than level 35 of the modified Airlie House Classification of diabetic retinopathy at the commencement of the study (Cunha-Vaz et al., 1998). They were examined by fundus photography, fluorescein angiography and vitreous fluorometry, at entry into the study and 1, 4 and 7 years after the initial examination. After 7 years follow-up a total of 22 of the 40 eyes had received photocoagulation. The eyes that needed photocoagulation were those that had higher vitreous fluorometry values at entry to the study and showed higher rates of deterioration. Abnormally high vitreous fluorometry values and their rapid increase over time were shown to be good indicators of progression and worsening of the retinopathy with need for photocoagulation.

Similar findings have been reported by Engler et al. in an 8-year follow-up study of type 1 diabetic patients (Engler et al., 1991). Initially, the patients were submitted to fundus photography and vitreous fluorometry for determination of the BRB permeability. After 8 years the patients were re-examined. A positive correlation between a high initial permeability value and an unfavorable clinical course, using photocoagulation as the outcome parameter, was found. In summary, in patients showing the same retinal morphology, high permeability of the BRB indicates a particular phenotype characterized by an unfavorable course of disease (Waltman, 1989).

It appears, therefore, that an alteration of the BRB, measured by vitreous fluorometry, is an early finding in diabetic retinal disease and correlates well with progression and worsening of the retinopathy.

One major limitation of the available commercial instrumentation for V.F. was associated with the fact that the permeability of the BRB is measured as an average over the macular area. Accurate mapping of localized changes in the permeability of the BRB would be beneficial for early diagnosis, to explain the natural history of retinal disease, and to predict its effect on visual acuity.

We have recently developed a new method of retinal leakage mapping, the retinal leakage analyzer (RLA), that is capable of measuring localized changes in fluorescein leakage across the BRB while simultaneously imaging the retina (Fig. 3). The instrument is based on a confocal scanning laser ophthalmoscope that was modified into a confocal scanning laser fluorometer (Lobo et al., 1999).

Two types of information are obtained simultaneously, distribution of fluorescein concentration (retina and vitreous) and fundus image. This simultaneous acquisition is crucial because it allows a direct correlation to be established between the maps of permeability and the morphological information.

It is now possible to follow the natural history of focal alterations of the BRB occurring in the initial stages of the disease and to identify their location and measure the changes over time, while examining their association with the main morphological changes occurring in the diabetic retina, such as microaneurysms, capillary closure and retinal edema.

2.3. Retinal blood flow and capillary closure

Retinal ischemia due to vascular closure develops relatively early in the course of diabetic retinopathy and is attributed to changes in vascular autoregulation and microthrombosis formation. Retinal blood flow changes are considered to lead to the development of poor perfusion facilitating microthrombosis formation (Boeri et al., 2001).

The control of blood flow through the retina depends on changes in the ophthalmic artery caliber (which has sympathetic innervation) or humoral and local factors. Retinal vessels have no sympathetic nerve supply (Malmfors, 1965). Therefore, local factors play a dominant role. Retinal vessels are particularly responsive to changes in arterial pO_2 and to a lesser extent, to changes in pCO_2 (Kohner et al., 1975; Kohner, 1977). The response to altered demand by the tissues is mediated through the "autoregulatory" adaptation of the blood vessels and blood flow.

Alterations in retinal blood flow have been identified in the different stages of the progression of retinopathy. In patients with mild retinopathy and using a two-point fluorophotometry technique, we found an increase in retinal arteriolar velocity (Cunha-Vaz et al., 1978). This finding was confirmed by Cuypers et al. (2000) using laser Doppler flowmetry. Other authors have, however, registered a decrease in retinal blood flow. Sullivan and associates (Sullivan et al., 1990) found reduced blood

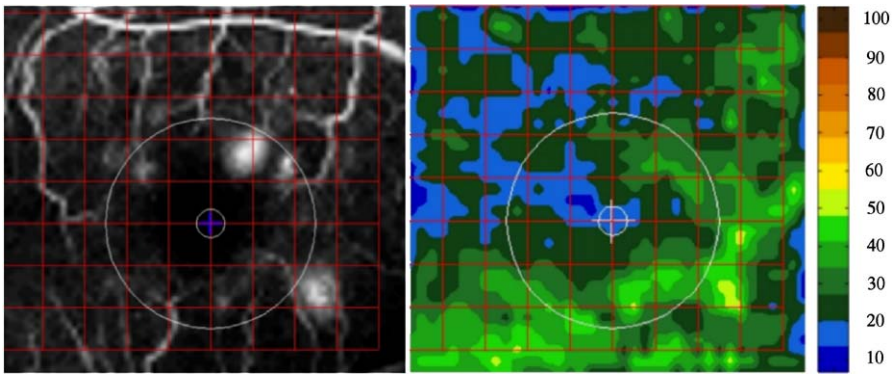

Fig. 3. Macula from a patient with diabetes type 2. Left: fluorescein angiography obtained by scanning laser ophthalmoscope. Right: retinal leakage analyzer BRB permeability map (Rlmap) in a false color-code map. Two-concentric circles of 100 and 750 μm radii centered on the fovea are shown on the figure. Note that not all hyperfluorescent areas in the left picture correspond to sites of increased fluorescein leakage into the vitreous, well depicted in the right.

flow when the glucose levels remained low and stable, increasing only in association with high glucose levels. In more advanced stages of retinopathy, the results also conflict, with some authors reporting decreases in retinal blood flow (Michelson et al., 2001) and others reporting increases (Bursell et al., 1996).

One of the major problems associated with these measurements is their technical complexity and variability.

Cuypers et al. (2000) used the Heidelberg Retina Flowmeter (HRF; Heidelberg Engineering, Dossenheim, Germany) on a series of eyes from patients with either type 1 or type 2 diabetes. They included in the study patients with all grades of retinopathy. They considered the methodology to be reliable.

We have examined with the HRF a series of eyes without clinical signs of retinopathy of subjects with type 2 diabetes and compared our findings with a control healthy population, after examining the reproducibility of the different softwares available and different examination methodologies (Ludovico et al., 2003).

We have, in this study, used the HRF, realizing that this technique is technically restricted to perform reliable measurements of blood flow in small vessels of the retinal capillary superficial layers. Retinal capillary blood flow measurements performed in the papillomacular area using the whole-scan analysis and the automated full-field perfusion image analysis proposed by Michelson et al. (1998) showed acceptable reproducibility in both healthy and diabetic eyes. This methodology involves automatic subtraction of large vessels and takes into account the heartbeat associated pulsation, artificial movements and local variations in brightness of the fundus.

When comparing the retinal capillary blood flow measurements obtained from the papillomacular area with the HRF using the automated full-field perfusion image analysis method in diabetic eyes with preclinical retinopathy and healthy control eyes, the retinal blood flow was increased in the diabetic eyes. However, when analyzing the results obtained in each eye it became clear that this increase in retinal blood flow varied markedly between different patients. Five of the 10 diabetic patients showed clearly abnormal increases in retinal capillary blood flow, i.e., with four of them presenting values higher than the mean +2SD of the values registered for all parameters (volume, flow and velocity) in the normal control group. The other five diabetic patients showed values within the normal range.

These findings may have particular relevance. They may explain the conflicting reports in the literature and indicate that changes in retinal capillary blood flow are an early alteration in the diabetic retina but do not occur in the same degree or at the same time in every retina. They may develop as a result of other retinal alterations and may be of particular value by identifying the eyes that are at risk for progression of the retinopathy and this way indicating different phenotypes of diabetic retinopathy.

In this study, the increases in retinal capillary blood flow registered in 5 of the 10 diabetic eyes with preclinical retinopathy did not show any clear correlations with level of metabolic control (HbA_{1C}), blood glucose values on the day of the examination, duration of the disease, blood pressure levels or other systemic variables, in agreement with the observations of Cuypers et al. (2000).

There are several possible explanations for an increase in capillary blood flow in diabetes (Kohner et al., 1975). It could indicate shunting, an increase in capillary diameter or capillary recruitment.

The flow of red blood cells through retinal capillaries is modulated by intravascular and extravascular factors. The intravascular pressure gradient between the precapillary arteriole and the postcapillary venule is

considered the most important regulator of capillary flow. In this study, all patients with type 2 diabetes had similar and acceptable levels of blood pressure. On the other hand, an increase in capillary diameter is considered to have a relatively small effect on the pressure gradient and, therefore, results in a small decrease in capillary flow. Shunting phenomena or capillary recruitment are the most likely candidates to explain the marked increase in capillary red blood cell flow observed in five of the 10 eyes of patients.

It is thought that under normal physiological conditions most retinal capillaries are perfused by both plasma and red blood cells. Fluorescein angiographic studies indicate that retinal capillaries are continuously perfused. However, the fluorescein method does not distinguish between flow of plasma and flow of plasma and red cells together.

It is accepted, that in the brain, in a small fraction of capillaries, red blood cell perfusion may stop for brief periods—not longer than a few seconds—indicating some degree of intermittence of red blood flow in the capillaries, i.e., plasma skimming.

Whether capillaries open and close at rest, and during adaptation of capillary blood flow to changing metabolic needs, is still a matter of controversy. Functional "thoroughfare channels" or preferential capillaries with high resting flow have been proposed to play a central role in microcirculation of the brain, surrounded by other capillaries, characterized by slow resting flow which could be recruited when the tissue blood supply is challenged (Hasegawa et al., 1967).

Finally, another possible alternative is that in the retinal capillaries plasma flow is continuous but red cells travel through only some capillaries at all times. In this case, capillary recruitment would be a natural response to increased metabolic demands by the retinal tissue in diabetes (Kageman et al., 1999) or a situation of relative hypoxia as proposed for the diabetic retina (Keen and Chlouverakis, 1965).

It is possible that the eyes which show increased capillary blood flow, thus apparently creating conditions for more rapid and progressive damage of the capillary walls are at a special risk of progression to retinopathy.

In this pilot study, there were no apparent correlations between the capillary blood flow alterations and the metabolic alteration of the diabetic patients at the time of the examination. This observation suggests that the capillary blood flow alterations registered are not acute and transitory but may indicate a more permanent status of the retinal circulation and characterize a specific retinopathy phenotype, which appear to be independent of age, metabolic control or duration of diabetes.

Our observations indicate that in some diabetic eyes, even before the development of visible retinopathy, there is (probably due to local factors) a marked increase in retinal capillary blood flow with the maximal utilization of the retinal capillary net, whereas others do not show this circulatory response.

This increase in blood flow may contribute to endothelial damage and establish the appropriate conditions for microthrombosis formation.

In diabetes, plasma constituents are also affected and red cells may be altered. Changes in blood viscosity increase the chances of microthrombosis formation, vessel damage and capillary closure.

Through abnormalities in clotting and the fibronolytic system in diabetes play certainly a role in retinal capillary closure it is unlikely that they initiate the process of diabetic retinal vasculature disease. The posterior pole of the retina is affected initially in diabetes in clear contrast to the peripheral involvement which characterizes the retinopathies resulting from blood disorders, like sickle-cell disease, macroglobulinemia and multiple myeloma (Cunha-Vaz, 1978).

2.4. Neuronal and glial cells changes. Retinal thickness measurements

We have stated previously that the simplest paradigm to explain increased capillary permeability and the advent of capillary closure centers on vascular endothelium and pericytes. There are, however, a number of reports showing changes in the neuronal and glial cells of the retina in diabetes very early in the course of the disease (Lorenzi and Gerhardinger, 2001). This is clearly of major potential importance and it may indicate at least a contributory role in the development of the microangiopathy.

Reports of electroretinographic changes in diabetic patients with demonstrable vascular lesions date back to the 1960s and have been confirmed by several authors (Ghirlanda et al., 1997) who found the electroretinographic abnormalities to originate in the ganglion and inner nuclear layers. Studies mostly performed in streptozotocin-induced diabetic rats have identified changes in the neuroglial elements of the inner two-thirds of the retina. Whether the neuroglial abnormalities induced by diabetes eventually contribute to the development of vascular pathology is still not known at present.

Hard and soft exudates are frequent components of the clinical picture of diabetic retinopathy. They are considered to result from increased vascular permeability and capillary closure but are definitely associated with neuronal and glial damage.

In the rat retina, most of the neurons containing neuronal nitric oxide synthase (NOs) appear to be from amacrine cells which are closely related to the retinal vasculature. The number of these *n*-NOs containing cells was found to decrease by 32% as early as 1 week after

the induction of STZ-diabetes (Darius et al., 1995). This reduced availability of nitric oxide may play a role by inducing localized changes in blood flow. Such alterations may also be compounded by increased endothelin-1 and endothelin-3 levels, which have been observed in the neural cells of the inner retina 2–4 weeks after STZ-diabetes (Deng et al., 1999). It is noteworthy that increased endothelin levels may be induced by high glucose through activation of protein kinase C (PKC) (Takagi et al., 1996).

In the retina of diabetic rats another major alteration observed only 1 month after STZ-diabetes is a 10-fold increase in the frequency of apoptosis (Takagi et al., 1996). The majority of apoptotic cells appeared to be ganglion cells.

Although the extent of abnormalities in the neural retinal cells in human diabetes appears to be less conspicuous, there is also clear evidence of their occurrence.

Namely, the phenomenon of early neuroretinal apoptosis appears to be less prominent in human diabetes than in the STZ-diabetic rats. However, the issues of early and extensive neural apoptosis in human diabetes and whether it precedes microangiopathy remain to be settled.

Regarding the involvement of the glial cells in diabetes there is also a wealth of information. Both Muller cells and astrocytes envelope neurons the initial segments of the ganglion cell axons, and blood vessels. Specifically, the inner layer of retinal capillaries is enveloped by both astrocytic and Muller cell processes, while only the latter provide most of the glial wrapping to the outer layer of the retinal vasculature (Holländer et al., 1991).

Muller cells have, indeed, characteristics that make them potential targets of diabetes and potential contributors to retinopathy. Muller cells are the primary site of glucose uptake and phosphorylation in the retina (Poitry-Yamate et al., 1965). They are endowed with Glut1 and metabolize glucose intensely through glycolysis to produce lactates that fuel neuronal metabolism and are the primary site of glycogen storage and metabolism in the retina. Muller cells are also primarily involved in the transformation of glutamate in the retina and in the acquisition of barrier properties by the endothelial cells of the BRB.

Recent evidence suggests that retinal glial, and Muller cells in particular, are affected early in the course of both experimental and human diabetes. There are reports demonstrating increased expression of glial fibrillary acidic protein (GFAP) and reduced ability to convert glutamate into glutamine in diabetic rat retinas. Glutamate excitotoxicity may occur in the diabetic retina as a consequence of Muller cell dysfunction (Lieth et al., 1998). It is still a matter of controversy if these changes are preceded by an increase in capillary permeability (Rungger-Brändle et al., 2000) and the early alteration of the BRB.

It is interesting to note that the overexpression of GFAP appears to be selective and is not likely to reflect an increased number of Muller cells.

Thus, in both human and experimental diabetes, the circumstances of increased retinal GFAP point to altered regulation of gene expression. This could be due to selective transcriptional effects of high glucose or other metabolic abnormalities on the GFAP gene, or be an element of more generalized changes in Muller cells fielding a specific "reactive" phenotype.

Diabetic macular edema is an important alteration occurring in the initial stages of diabetic retinal disease. Its importance is due to its association with loss of vision. Based on WESDR data, it was estimated (as of 1993) that of approximately 7,800,000 people with diabetes about 84,000 North Americans would develop proliferation retinopathy and about 95,000 would develop sight loss from macular edema over a 10-year period (Klein et al., 1995a, b).

Edema of the retina is any increase of water of the retinal tissue resulting in an increase in its volume, i.e., because of the structural organization of the retina, an increase in its thickness. Macular edema is, therefore, edema of the retinal tissue located in the macular area.

This increase in water content of the retinal tissue may be initially intracellular or extracellular. In the first case, also called cytotoxic edema, there is an alteration of the cellular ionic exchanges with an excess of Na^+ inside the cell. In the second case, also called vasogenic edema, there is predominantly extracellular accumulation of fluid directly associated with an alteration of the BRB (Cunha-Vaz and Travassos, 1984).

The clinical evaluation of macular edema has been characterized by its subjectivity. Direct ophthalmoscopy may show only an alteration of the foveal reflexes. Stereoscopic fundus photography and slit-lamp biomicroscopy play an important role demonstrating changes in retinal volume in the macular area but they are dependent on the observer experience and the results do not offer a true measurement of the volume change. Furthermore, the interpretation of the extent and type of macular edema varies markedly between different observers (Kylstra et al., 1999).

Recently, new techniques have become available that measure objectively retinal thickness.

Optical imaging instruments, like the retinal thickness analyzer (RTA, Talia Technology, Ltd,) and optical coherence tomography (OCT, Humphrey Instruments), have been proposed as powerful tools for the objective assessment of macular edema. Both techniques, which are able to measure retinal thickness and rapidly generate thickness maps at the posterior pole, are noninvasive and noncontact procedures. Another instrument, the Heidelberg retina tomograph (HRT,

Heidelberg Engineering) is a scanning laser ophthalmoscope that is able to measure retinal edema indirectly by performing a topographic assessment of an unevenly raised "retina" thus offering a map of relative increases in retinal thickness (Ang et al., 2000).

The RTA is a quantitative and reproducible method to evaluate retinal thickness. The variability in the measurements obtained in normal subjects was reported as 8% by Shahidi and co-workers (Shahidi et al., 1990).

The principle of the RTA is based on projecting a thin He–Ne laser (543 nm) slit obliquely on the retina and viewing it at an angle. The separation between the reflections (and scatter) from the vitreoretinal interface and the chorioretinal interface is a measure of the retinal thickness. On the other hand, OCT provides cross-sectional tomographs of the retinal structure in vivo, in which optical interferometry is used to resolve the distances of reflective structures within the eye.

Low coherence light from a superluminescent diode source, operating at 840 nm (infrared light), is divided into two beams: one incident on the retina and the other incident on a translating mirror. The two reflected beams, one on the mirror and the other on retinal structures, are recombined and optical interference detected by a photodiode.

The reproducibility of the method in normal subjects was reported as 7% by Ang and co-workers (Ang et al., 2000).

In recent studies performed with the RTA and OCT, we chose to compare 5 regional measurements of retinal thickness (Pires et al., 2002).

Two normal populations volunteered to participate as age matched control groups for RTA and OCT and reference maps were computed using the mean + 2SD.

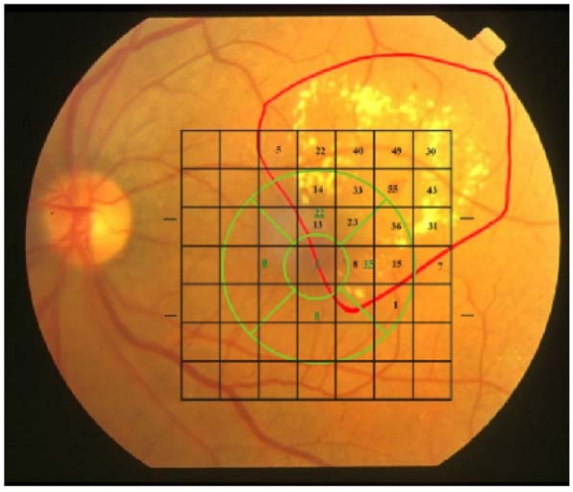

Fig. 4. The RTA and OCT identify well the retinal edema outlined by stereofundus photography. Numbers in black correspond to % increases in retinal thickness obtained using the RTA, whereas numbers in green represent % increases in retinal thickness obtained with OCT.

Measurements of retinal thickness obtained with the RTA or the OCT which were higher than the ones in these reference maps were expressed as % increases over the reference normal values (Lobo et al., 2000).

We examined three groups of eyes from subjects with diabetes type 2: (1) with preclinical retinopathy; (2) with mild to moderate nonproliferative retinopathy; (3) with mild clinically significant macular edema according to the ETDRS guidelines, edema identified by stereofundus photography.

In group 1, RTA detected abnormal increases in 86% of the diabetic eyes examined, with increases ranging from 0.3% to 73.5% over the normal mean value + 2SD. OCT detected retinal thickness increases in only 11% of the same series of eyes.

This study showed that there are localized areas of retinal edema, i.e., areas of abnormal increase in retinal thickness, occurring in the macula in the initial stages of diabetic retinal disease. The RTA, in this study, appeared to be able to detect localized increases in retinal thickness in the diabetic retina well before OCT.

Comparable results have been reported by Shahidi et al. (1991) and Hee et al. (1998). Shahidi et al. (1991) observed that stereofundus photography did not identify locations with mild or localized thickening demonstrated by the RTA. Hee et al. (1998) using the OCT only detected increases in foveal thickness in 3.6% of a series of 55 eyes with no visible retinopathy.

In another group of patients, with mild to moderate nonproliferative diabetic retinopathy, the RTA detected again larger increases, reaching values as high as 56.5% and 73.5% over the normal value + 2SD in eyes graded 20 and 35 of the Wisconsin grading scale, respectively. It was also clear from this study that the presence of localized areas of retinal edema identified by the RTA is not a constant finding in the diabetic retina, as a number of eyes, 14%, remained edema-free. It is to be noted that no clear correlation could be found between the extent of the edema and the retinopathy grading, at least in these initial stages of the retinopathy.

In the third study, we examined with the RTA and OCT a series of eyes of patients with diabetes type 2 presenting clinically significant macular edema as defined by stereofundus photography according to ETDRS characteristics (Fig. 4).

In the central macula, the RTA measured increases in retinal thickness higher than the mean + 2SD of a healthy control population in 21 of the 25 eyes with CSME characteristics (84%). There were, therefore, four eyes with a diagnosis of retinal edema on stereofundus photography which did not show increases in retinal thickness with the RTA. These four eyes, however, showed, the presence of isolated hard exudates, confirming the recent observations of Storm et al. (2002) indicating that the presence of hard exudates may mislead fundus photography graders into assuming the

presence of retinal edema. If these four eyes were excluded, the RTA detected increases in retinal thickness in 21 of the 21 eyes (100%) identified by fundus photography, whereas the OCT measured increases in retinal thickness in only 12 of the 21 eyes that had edema identified by fundus photography in the central macular area (57%). These findings are in agreement with the observations of Zeimer (1998) who reported an agreement of 78% between the RTA and stereophotography, with disagreements generally associated with the presence of hard exudates.

The eyes included in this study had characteristically good visual acuity and belonged to the category of relatively mild CSME. The OCT examination did not show in any of these eyes, the presence of cyst-like structures, large collections of fluid or signs of disorganization of the retinal structure, thus confirming the mild nature of the retinal edema.

Measurements of retinal thickness are particularly promising because of their quantitative nature and provision of permanent records of the location and degree of thickening.

Comparing the two techniques to measure retinal thickness, the RTA appears to be particularly appropriate to measure changes in retinal thickness in eyes with clear media and when these changes are minimal. We consider it to be an extremely promising tool in evaluating quantitatively the changes in retinal thickness before the development of cystoid macular edema, and when an early therapeutic intervention may be more effective. OCT, on the other hand, uses a unique cross-sectional scanning mode offering highly accurate anatomic representation of the retina, which is particularly useful when the retinal edema is associated with other pathologies. OCT, in our experience, is particularly informative when there are changes in the retinal architecture, namely through the formation of cyst-like spaces due to localized intraretinal fluid accumulation, or vitreous fraction is suspected. OCT is also to be preferred when there is some degree of cataract formation or the eyes have an implanted intraocular lens.

In our experience both methods are well accepted by the patients and can be performed with little discomfort, giving reliable quantitative information on the size and location of macular edema.

Measurements of retinal thickness offer an objective evaluation of retinal edema and show that localized areas of retinal edema are a frequent finding in the diabetic retina in the initial stages of nonproliferative retinopathy in subjects with diabetes type 2.

It is still not clear, however, if the retinal edema occurring in the initial stages of diabetic retinopathy is mainly vasogenic, due to the BRB alteration and what is the relative role played by cytotoxic damage of the neuronal and glial cells of the retina.

2.5. Multimodal macula mapping

The initial changes occurring in the diabetic retina involve the macula and an alteration of the macula will, sooner or later, affect visual acuity. Diabetic macular edema is a major contributor to visual loss because of its preferential location to the macula. Characterization of alterations occurring in the macula since their earliest stages is, therefore, absolutely fundamental to follow disease progression and preserve good visual acuity in diabetes.

There are a variety of diagnostic tools and techniques to examine the macular region and to obtain information on its structure and function. The different methods available offer different perspectives and fragmentary information. It has been our objective, in recent years, to combine different methodologies and to obtain maps of the alterations occurring in the macular region of the retina.

Detection devices for obtaining information for macula mapping are numerous and varied, often complementing one another with differing degrees of invasiveness, accuracy and object of measurement. Some chart anatomy whereas others measure an aspect of physiology. Together, they can combine structure and function. Our research group has been developing methods to combine and integrate data from fundus photography, angiographic images (scanning laser ophthalmoscope—fluorescein angiography), maps of fluorescein leakage into the vitreous (scanning laser ophthalmoscope—retinal leakage analyzer), maps of retinal thickness and maps of visual function (automated perimetry—humphrey field analyzer HFA II 750) of the macular area to achieve multimodal macula mapping (Lobo et al., 1999, 2000; Bernardes et al., 2002). Scanning laser ophthalmoscopy (SLO) produces high-resolution images using much less light for illumination of the fundus than used for conventional photography. High contrast images of the foveal and perifoveal structures are produced with this technique using directly reflected light. In confocal scanning laser ophthalmoscopy (CSLO), a laser beam illuminates an area of the eye fundus. A confocal stop placed in front of the detector rejects most of the light coming from both anterior and posterior planes. A set of moving mirrors allows the scanning of an area of interest. In SLO imaging, a laser beam illuminates an area of the ocular fundus, forming a rectangular pattern (raster) on the retina. The light reflected from each retinal point is captured by the detector. Thus, a point-by-point video image is constructed, with each retinal point corresponding to a point on the monitor screen. SLO, because of its monochromatic wavelength emission, minimizes scattering and chromatic aberration. This feature of SLO increases contrast and improves visibility

as compared with slitlamp biomicroscopy and fundus photography.

SLO can also be used to perform high-resolution fluorescein angiographies. The contrast of the image allows acquisition of high-quality morphological information on the retinal vasculature. A system combining SLO with Doppler flowmetry provides noninvasive evaluation of regional blood flow. We have been able to develop the retinal leakage analyzer, for measuring and mapping the permeability of the BRB based on CSLO system (Lobo et al., 1999). The combination of two data sets, angiographic and permeability mapping, obtained simultaneously using the same instrumentation, provided a good definition of landmark references of the macula, giving simultaneously functional and morphological information, and in this way was an important step in the development of multimodal mapping.

To integrate the information form the above sources, a common reference has to be established. The reference fundus image may be given by the RLA or by fundus digital images, when using the RLA. To compute the center of the fovea, the image is compressed with a LOG function. This produces an image with a marked difference between the FAZ and the remaining image. A cross-correlation function is computed between the compressed image and a 2D Gaussian function. The result allows the determination of the center of the fovea, since the shift in the maximum of the cross-correlation to its center equals the shift of the center of the fovea to the center of the fundus image.

We have been able to combine in multimodal macula mapping, information on structure and function by integrating data from fluorescein angiography, retinal leakage analysis (Fig. 5), and retinal thickness analysis. Other available detection devices that may be used for macula mapping include laser Doppler retinal flowmetry using the Heidelberg Retina Flowmeter, indocyanine green angiography (ICG), electrophysiology (ERG), autofluorescence mapping and OCT (Fig. 6). Each one of these methods adds more information and appears as potentially valuable tools for evaluating the structure and function of the retinal macula.

When examining diabetic macular edema, OCT and HRF methodologies become particularly interesting especially when the diabetic macular edema is associated with some degree of vision loss. The HRF appears to be particularly useful and reliable to follow changes in capillary perfusion in the macular area and we are

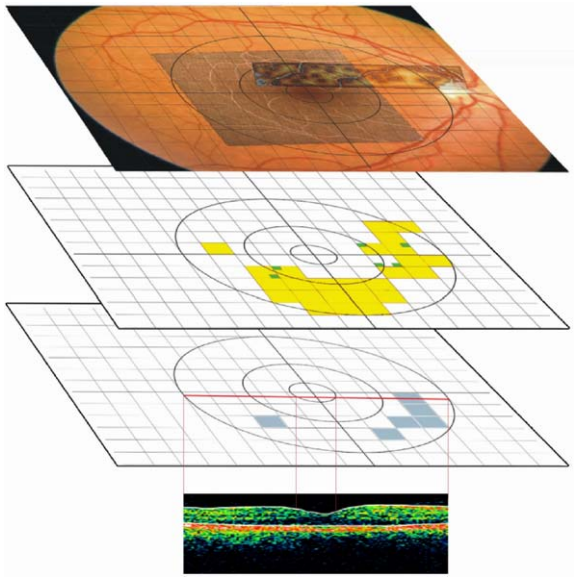

Fig. 6. This multimodal image shows the integration of morphology, blood flow, thickness, leakage, visual function and OCT cross-section image as a stack of images. The top image contains information on morphology and flow measurements. The second shows thickness changes and identifies leaking sites. The third represents visual field changes. Finally, in the bottom, the OCT cross-sectional image.

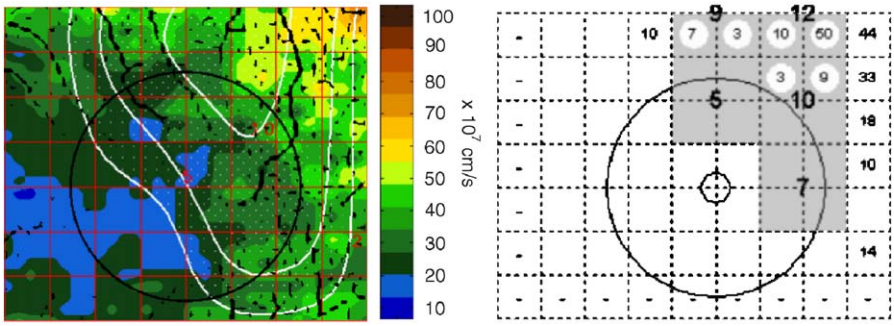

Fig. 5. Multimodal macula mapping of an eye with mild NPDR showing localized increases in leakage and retinal thickness. Left: the background represents the leakage using a false color-code. Units are $\times 10^{-7}\mathrm{cm\,s^{-1}}$. Percentage of increased thickness is represented in white dots with varying density. Lines in white represent equal percentage of increase in thickness. Black lines and dots represent the retinal vasculature. Right: the gray areas represent increased retinal thickness. Percentages of increase are represented in bold and intersecting lines. Each value represents four adjacent squares. Percentages of increased leakage are represented by numbers in the squares. Also represented are two concentric circles of 100 and 750 μm radii centered on the fovea.

examining closely its value to indicate progression of ischemia.

Electrophysiological methods have been recently proposed for retinal mapping with the introduction of the multifocal electroretinography (mERG). The multifocal ERG technique was developed by Sutter (2001) and can produce 100 or more focal responses from the cone-driven retina. The display usually contains either 61 or 103 hexagons, although 241 hexagons have been used in a few experiments to obtain higher spatial resolution. With the 103 elements display, there is no guarantee that a hexagon will fall entirely within the optic disc. With a display of 241 elements, at least one hexagon should fall entirely within the disk if steady fixation is maintained. In conclusion, the mERG method offers attractive information on the electrical functions of the retina, but improved spatial resolution is necessary for useful macula mapping.

3. Characterization of retinopathy phenotypes

It is well recognized that duration of diabetes and level of metabolic control are major risk factors for development of diabetic retinopathy.

However, these risk factors do not explain the great variability that characterizes the evolution and rate of progression of the retinopathy in different diabetic individuals. There is clearly great individual variation in the presentation and course of diabetic retinopathy. There are many diabetic patients who after many years with diabetes never develop sight-threatening retinal changes, maintaining good visual acuity. There are also other patients that even after only a few years of diabetes show a retinopathy that progresses rapidly and does not respond to laser photocoagulation treatment.

We have recently performed a prospective 3-year follow-up study of the macular region, in 14 patients with type 2 diabetes mellitus and mild nonproliferative retinopathy, using multimodal macula mapping (Lobo et al., 2004).

In a span of 3 years, eyes with minimal changes at the start of the study (levels 20 and 35 of ETDRS-Wisconsin grading) were followed at 6-month intervals in order to monitor progression of the retinal changes.

The most frequent alterations observed were, by decreasing order of frequency, RLA-leaking sites, areas of increased retinal thickness and microaneurysms/hemorrhages.

RLA-leaking sites were a very frequent finding and reached very high BRB permeability values in some eyes. These sites of alteration of the BRB, well identified in RLA-maps, maintained, in most cases, the same location on successive examinations, but their BRB permeability values fluctuated greatly between examinations, indicating reversibility of this alteration (Fig. 7).

There was, in general, a correlation between the BRB permeability values and the changes in HbA_{1C} levels occurring in each patient. This correlation was particularly clear when looking at eyes that showed, at some time during the follow-up period, BRB permeability values within the normal range. A return to normal levels of BRB permeability was, in this study and in each patient, always associated with a stabilization or decrease in HbA_{1C} values.

The frequent finding of RLA-leaking sites in these 14 eyes confirms previous reports using fluorescein angiography (Wise et al., 1971), vitreous fluorometry (Cunha-Vaz et al., 1985a, b) and retinal leakage analysis (Lobo et al., 1999), which show that alteration of vascular permeability is one of the most frequent alterations occurring in the initial stages of diabetic retinal disease.

Areas of increased retinal thickness were another frequent finding in these eyes. They were present in every eye at some time during the follow-up and were absent, at baseline, in only two of the 14 eyes. This confirms previous observations by our group (Lobo et al., 1999) and by others (Fritsche et al., 2002).

However, the areas of increased retinal thickness varied in their location over subsequent examinations and did not correlate with changes in HbA_{1C} levels. They may represent a delayed response in time to other changes occurring in the retina, such as increased leakage, as suggested previously (Lobo et al., 1999).

The areas of increased retinal thickness may, indeed, represent in most cases, zones of extracellular edema, an interpretation supported by the frequent shift observed in their location.

Increased rates of red-dot accumulation were found in eyes that showed more red-dots at baseline and higher values of BRB permeability during the study. By combining different imaging techniques, multimodal imaging of the macula made apparent three major evolving patterns occurring during the follow-up period of 3 years: *Pattern A* included eyes with reversible and relatively little abnormal fluorescein leakage, a slow rate of microaneurysm formation and a normal FAZ. This group appears to represent eyes presenting slowly progressing retinal disease; *Pattern B* included eyes with persistently high leakage values, indicating an important alteration of the BRB, high rates of microaneurysm accumulation and a normal FAZ. All these features suggest a rapid and progressive form of the disease. This group may identify a "wet" form of diabetic retinopathy, and; *Pattern C* included eyes with variable and reversible leakage and an abnormal FAZ. This group is less well characterized considering the small number of eyes that showed an abnormal FAZ. It may be that abnormalities of the FAZ may occur as a late development of groups A and B or progress rapidly as a specific "ischemic" form (table).

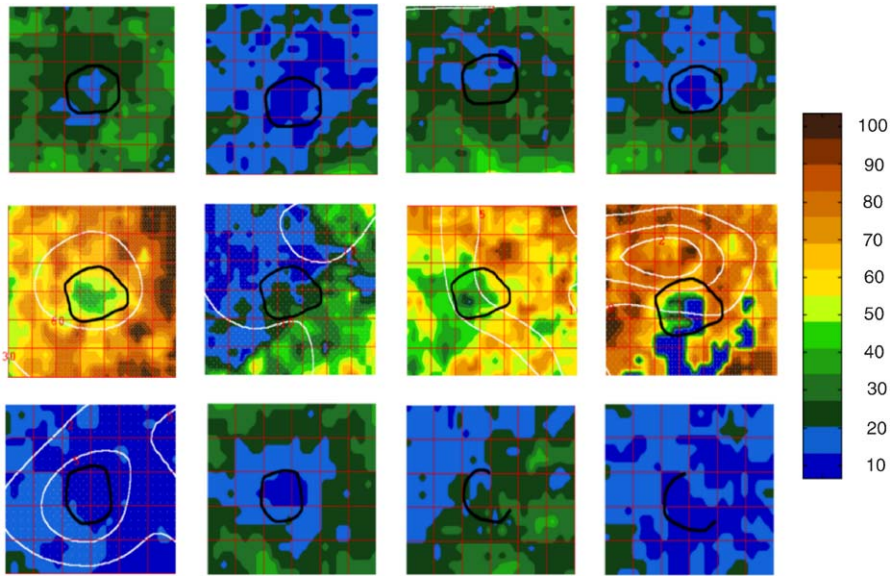

Fig. 7. Multimodal images from with 0, 12, 24 and 36 (left to right) showing for each visit the FAZ—black contour—retinal leakage analyzer results and retinal thickness analyzer results. The retinal leakage analyzer color-coded maps of the BRB permeability indexes are shown; retinal thickness analyzer views show white dot density maps of the percentage increases in retinal thickness. Top row: pattern A. Note the little amount of retinal leakage over the 4 represented visits and the normal FAZ contour. This patient showed a slow rate of microaneurysm formation. Middle row: pattern B. Note the high retinal leakage showing a certain degree of reversibility and the normal FAZ contour. This patient showed a high rate of microaneurysm accumulation over the 3-year follow-up period. Bottom row: pattern C. Note the reversible retinal leakage and the development of an abnormal FAZ contour. This patient showed a high rate of microaneurysm formation.

We have now extended our observations after following for 2 years 59 patients with diabetes type 2 and mild NPDR. In this larger study these three different phenotypes were again clearly identified. The discriminative markers of these phenotypes were: red-dot formation rate, measurements of fluorescein leakage, signs of capillary closure in the capillaries surrounding the FAZ and duration of diabetes.

It must be realized that levels of hyperglycemia and duration of diabetes, i.e., exposure to hyperglycemia, are expected to influence the evolution and rate of progression tentatively classified in these three major patterns (Fig. 8).

If diabetic retinopathy is a multifactorial disease—in the sense that different factors or different pathways may predominate in different groups of cases with diabetic retinopathy—then it is crucial that these differences and the possible different phenotypes be identified (Grange, 1995).

Diabetes mellitus is a familial metabolic disorder with strong genetic and environmental etiology. Familial aggregation is more common in type 2 diabetes than in type 1 diabetes. Rema et al. (2002) reported that familial clustering of diabetic retinopathy was three times higher in siblings of type 2 subjects with diabetic retinopathy. Presence or absence of genetic factors may play a fundamental role in determining specific pathways of vascular disease and, as a consequence, different progression patterns of diabetic retinal disease. It could

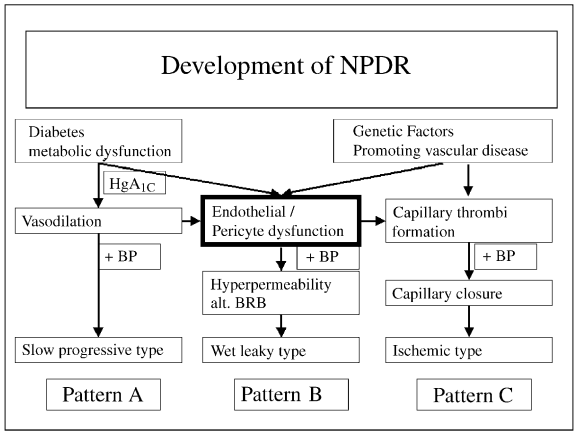

Fig. 8. Schematic development of nonproliferative diabetic retinopathy leading to the three different patterns proposed: A, B and C.

be that certain polymorphisms would make the retinal circulation more susceptible to an early breakdown of the BRB (type B) or microthrombosis and capillary closure (type C). The absence of these specific genetic polymorphisms would lead to an evolving pattern of type A.

It is clear from this study and from previous large studies such as the Diabetes Control and Complications Trials group (DCCT) (2002), and UKPDS (Stratton et al., 2001) that hyperglycemia plays a determinant role in the progression of retinopathy. It is interesting to note

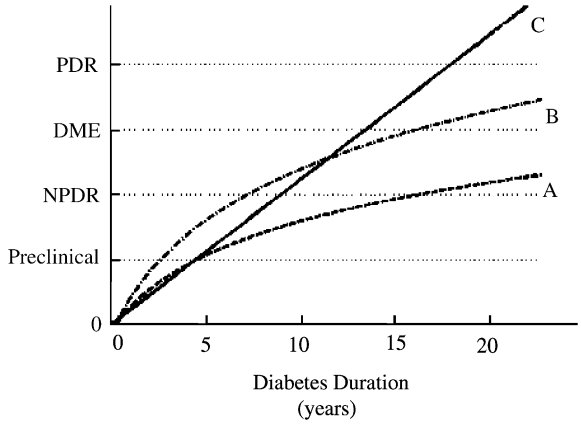

Fig. 9. Representation of different patterns of progression of different diabetic retinopathy phenotypes.

that HbA_{1C} levels are also largely genetically determined (Snieder et al., 2001).

An interesting perspective of our observations, analyzed under the light of available literature, depicts diabetic retinopathy as a microvascular complication of diabetes mellitus conditioned in its progression and prognosis by a variety of different genetic polymorphisms, and modulated in its evolution by HbA_{1C} levels, partly genetically determined and partly dependent on individual diabetes management. The interplay of these multiple factors and the duration of this interplay would finally characterize different clinical pictures or phenotypes of diabetic retinopathy (Fig. 9).

The ultimate goal, therefore, should be the characterization of relationships between genetic factors (represented by distinct genotypes) and their medically significant expression (distinct diabetic retinopathy phenotypes). Our observations of a 3-year prospective study of eyes with mild nonproliferative diabetic retinopathy of patients with type 2 diabetes mellitus, suggest three different phenotypes of diabetic retinopathy: a "wet" or "leaky" type, an "ischemic" type, and finally, an apparently more common, slow progression type.

4. Directions for future work

4.1. Candidate phenotype/genotype correlations

It is recognized that polymorphic variability in the genetic make-up of an individual can profoundly influence the expression of a gene and its response to environmental factors. As we predict that the impact of single common mutations on RD development will be modest (increasing relative risk (RR) by 20–40% at most), the main issue of clinical relevance is whether the conferred risk of such a mutation is very much higher in some population subgroups. To be clinically useful in a risk algorithm we might require for any factor to have a RR of 2 or greater (Humphries et al., 2001).

Such subgroups might be those carrying a second important mutation in another gene and such individuals might be identified using conventional genetic strategies. Alternatively, one might identify individuals exposed to a given environment which amplifies the risk associated with that gene (i.e., gene–environment interaction).

Diabetic retinopathy shows familial aggregation and variation in disease severity which is not explained by environmental, biochemical or biological risk factors alone.

There are substantial variations in onset and severity of retinopathy in different patients which are independent of the duration of diabetes and level of glycemic control.

A relatively large number of candidate genes have been examined in patients with diabetes but clear genotype–phenotype associations have not yet been identified.

One of the major problems is associated with poor characterization of different retinopathy phenotypes. It is fundamental before embarking in a search for candidate genes to define clinical phenotypes characterized by specific patterns of severity and progression of DR. It is clear that it is necessary to identify first and well the DR phenotypes that are associated with rapid progression of the retinopathy to severe forms of the disease, such as macular edema and proliferative retinopathy. Only then, studies on candidate genes are worth pursuing, involving appropriately well-defined subgroups of patients (Warpeha and Chakravarthy, 2003).

There are several physiological systems that are involved in maintaining retinal vascular health and disease can be predicted to develop resulting from a failure to maintain hemostasis. One, is the endothelial lining of the vessel wall with its role in maintaining the BRB, influencing vessel tone, maintaining normal wall structure and preventing thrombosis. Another, is the coagulation cascade. Other possibilities include the normal homeostatic systems which regulate short-term blood pressure and plasma and intracellular lipid metabolism.

Variations in the genes expressed in the aldose reductase pathway may influence microvascular susceptibility. Aldose reductase is strongly expressed in retinal pericytes and is also found in the vascular endothelium (Vinõres et al., 1993). It has been suggested that 7q35 is a susceptibility region for diabetic retinopathy by virtue of the aldose-reductase gene (AR2) (Patel et al., 1996).

In type 1 diabetes the strongest genetic risk component is localized within the major histocompatibility complex. The HLA region that is located on 6p21, has also been implicated as genomic region of interest for

susceptibility to retinopathy in both type 1 (Stewart et al., 1993) and type 2 diabetes (Serrano-Rios et al., 1983).

Glut transporter genes have been examined but no association between polymorphisms in the Glut 1 gene and retinopathy status was found.

Other candidate genes involved in cell communication and the extracellular communication have also been investigated without conclusive data (Warpeha and Chakravarthy, 2003). These authors and their group have paid special attention to the genes of endothelins (ET) and nitric oxide synthases (NOS). NOS and ET are counter regulatory and the NO/ET pathway is crucial to maintain the tone of the vasculature, which is delicately controlled by the balance in their expression. They identified microsatelite polymorphic markers in members of ET and NOS families as well as those of endothelin converting enzyme (ECE1).

Subjects with no retinopathy despite 15 years or more of diabetes (controls) and any subject with severe retinopathy regardless of duration (ETDRS level 50 or worse) were prospectively recruited into these studies. None of the polymorphisms studied in the NOS 1 or NOS 3 genes was significantly associated with cases or controls. However, studies on the NSO2A gene showed that a 14-repeat allele of a pentanucleolide polymorphism in the 5′UTR of NOS2A gene was protective against developing diabetic retinopathy in both patient populations (Warpeha et al., 1999). The authors suggest that when NOS3 expression is low in the diabetic retina induction of NOS2A may occur in an attempt to achieve homeostasis (Graier et al., 1993), possibly playing a crucial role in preventing or delaying pathological alterations in the microcirculation in diabetes.

There are indications that the vascular complication of diabetes are related with the formation of advanced glycated-end products (AGE). The glycation of proteins and lipids is a result of hyperglycemia and this effect of AGEs is mediated the AGE receptor (RAGE) which is regulated by a gene (Hudson et al., 2001).

Angiotensin-converting enzyme (ACE) is another important mediator of vasoconstriction and homeostasis; however, studies to date on genetic markers of members (Matsumoto et al., 2000) of this signaling pathway have not shown definitive evidence of direct genetic risk. It remains, however, an interesting candidate gene to play a role in DR. Clinical studies have shown that ACE inhibition may play a useful specific role in the management of DR.

Large interindividual differences in plasma ACE levels exist but are similar within families, suggesting a strong genetic influence. The human ACE gene is found on chromosome 17 and contains a restriction fragment length polymorphism consisting of the presence (insertion, I) or absence (deletion, D) of a 287 base pair "ALU" repeat sequence in intron 16 (Rigat et al., 1990). In 1992, the I/D polymorphisms of the ACE gene were reported to be associated with risk of myocardial infarction (Cambien et al., 1992) and this effect, though more modest then originally reported has been confirmed in larger studies (Keavney et al., 2000).

Glucose may itself be the mitigating environmental factor that induces expression of the polymorphism.

The situation on a complex and multifactorial disease such as diabetes favors the presence of gene–environment interactions. A key factor in the identification and study of gene–environment interaction is that an individual carrying such a mutation will develop the phenotype only if and when they enter the high risk environment. Thus, the mutation will cause a specific retinal vascular alteration, i.e., alteration of BRB or blood-flow changes in the presence of a specific environmental challenge. This classical "lack of penetrance" of a mutation will cause analytical problems and mis-phenotyping which will be particularly problematic with some sampling analytical designs. This "content-dependency" of a mutation (i.e., gene X environment effect) must be taken into consideration when analyzing associations between a candidate gene polymorphism and intermediate phenotypes.

Most of the results published indicate the presence of genetic determinants for resistance or susceptibility to vascular complications. However, there is evidence of problems in replicating results suggesting that the studies performed have been plagued with confounding factors.

The results of our research group on the characterization of different phenotypes of DR confirm that there are distinct morphological manifestations in DR with different subjects presenting different rates of progression and different evolution patterns. (Lobo et al., 2004). There is, now, evidence indicating that susceptibility to the late vascular complications of diabetes, such as retinopathy, depend, at least partly, on genetic factors (Wasgenknecht et al., 2001).

The risk of severe DR in the siblings of affected individuals is substantially increased (Leslie and Pyke, 1982). It is possible that the problems associated with identifying susceptibility genes for diabetic retinopathy is due mainly to the still accepted view that diabetic retinopathy is one uniform and homogenous disease. Specific types of more severe retinopathy may need to be identified before progress is achieved in this area of research.

Another factor that must be taken into consideration is duration of diabetes. Problems encountered may be minimized by selecting case subjects with short diabetes duration and control subjects with larger duration, or by adjusting to duration during analysis.

It is clear that future studies should focus on the need to characterize more accurately different phenotypes with respect to retinopathy status. We agree entirely

with Warpeha and Chakravarthy (2003) when they state that agreed international standards for data collection, particularly agreement on a minimum data set for the phenotyping of retinopathy in subjects with diabetes, would permit the pooling of data from the many studies with enhanced power to detect associations.

A classification of diabetic retinopathy based on both relevant genotypes and disease phenotypes is an ambitious goal. We believe that this route may help identify the particular form that threatens an individual patient and consequently offer an opportunity for specific and more effective therapies.

4.2. Relevance for clinical trial design

Studies such as the Diabetes control and complications trial (Diabetes control and complications trial Group, 2002), the United Kingdom prospective diabetes study (United Kingdom Prospective Diabetes Study, 1998), the diabetic retinopathy study research group (Diabetic Research Study Research Group, 1981) and the early treatment diabetic retinopathy study (Early Treatment Diabetic Retinopathy Study, 1991) validated methods now considered standard in treating diabetic retinopathy when it occurs, i.e., tight control of blood glucose levels to prevent retinopathy and laser photocoagulation to halt progression after development of CSME or proliferative retinopathy. However, despite the aim of tight blood glucose control and the use of retinal photocoagulation, blindness still occurs. Other forms of therapy targeted at the earliest stages of retinal disease, involving necessarily the demonstration of efficacy of a new drug are urgently needed and remain a priority for eye research.

One of the major problems lies in the limitations that characterize at present the accepted methods of retinopathy assessment, visual acuity changes and fundus photography. Visual acuity changes are detected too late in the course of the disease and fundus photography, as it has been used, in descriptive manner, is subjective and has been unable to characterize progression in the initial stages of the retinopathy. To make things more difficult it is well known that the course of retinopathy is not linear and lesions that appear on fundus photography may come and go. Apparent clinical improvement on fundus photography may, in reality, mask worsening of the disease.

It is crucial in order to design an appropriate clinical trial to test the efficacy of a drug, to identify not only the meaningful clinical endpoints but also the surrogate endpoints that may demonstrate efficacy of a drug in a realistic and feasible period of time (Cunha-Vaz, 2000b).

Approval of a drug to treat diabetic retinopathy must take into account that it must appeal to clinicians as being useful to their patients, which means that it must show efficacy to clinical endpoints that they can see in their patients and recognize "this is the patient I would use that therapy for".

It must be taken into account that the clinicians must understand what the drug is being used for and in which group of patients, so that they can explain the potential risks and benefits to their patients.

It is immediately clear that such process implies the validation of surrogate endpoints by the associated occurrence of hard clinical outcomes such as significant visual loss. It is here that the problem lies. Diabetic retinopathy progresses to irreversible stages of the disease with relatively little visual loss, and when macular edema or proliferative retinopathy are present, it becomes ethically mandatory to perform photocoagulation treatment.

The development of an effective drug must take into account the need to demonstrate efficacy on the earliest and reversible stages of diabetic retinal disease by demonstrating its effect on surrogate endpoints which can be followed for shorter periods of time. The assumption would be that those surrogate endpoints would ultimately be validated by association with more hard clinical outcomes, but that should not necessarily need to occur before provisional approval.

It is therefore an urgent priority to identify endpoints which people can accept as surrogates that are expected to be later validated in longer natural history studies.

The clinical endpoints that have been accepted in the past are: mean difference between groups in visual acuity of at least 3 lines in a ETDRS-Type chart, i.e., doubling of the visual angle; mean difference in visual field of at least 10 dB; reduction in percentage of patients with vitreous hemorrhage; reduction in percentage of patients with rubeosis; reduction in occurrence of retinal detachments, and need for photocoagulation treatment according to DRS and ETDRS guidelines (DRS, 1981; ETDRS, 1991). All of these are what may be called terminal endpoints. They only give indications about the late, irreversible, stages of diabetic retinopathy.

The candidates for surrogate endpoints in the initial stages of the retinal disease are not many: mean differences on the ETDRS retinopathy scale, reduction in fluorescein leakage, reduction in macular thickening and red-dot (microaneurysms/hemorrhages) counting.

The problem of using the ETDRS retinopathy scale lies in the fact that in the initial stages of the retinopathy even a two-step eye change means that we have to wait for an important worsening of the retinopathy and the presence of irreversible changes.

The second possibility, reduction in fluorescein leakage, measures one of the two main factors in the progression of the retinopathy, the alteration of the BRB permeability which is quantitative, allowing for precise evaluation of progression. It has, however, a major drawback, it involves fluorescein administration

and its widespread use must wait for the development of a reliable test involving oral fluorescein administration.

The third candidate, reduction in macular thickening by measuring the mean change with dedicated instrumentation, is a promising alternative. The measurements are reliable, and changes in retinal thickness are a direct indication of macular edema (Shahidi et al., 1994; Hee et al., 1995). The problem associated with this outcome lies in the variability of the evolution of macular edema and in the fact that macular edema is only one of the manifestations of diabetic retinal disease.

Finally, the fourth possibility, red-dot counting on fundus photographs, taking into account every new microaneurysms/hemorrhage according to their exact, specific location in the eye fundus is noninvasive and has the potential to become an extremely informative marker of the overall progression of diabetic retinal vascular disease. The rate of formation of new red-dots appears to be a direct indication of the progression of retinal vascular damage and is statistically correlated with the progression in fluorescein leakage, i.e., the alteration of the BRB and capillary closure (Torrent-Solans et al., 2003). By counting red-dots on digitalized fundus photographies, using appropriate software to identify the specific location of each red-dot, we may be able to measure the rate of progression of the two major factors in diabetic vascular retinal disease: vascular hyperpermeability and capillary closure.

It is crucial and urgent now to validate the last two candidates for surrogate endpoints, retinal thickness measurements and/or red-dot formation rates both using noninvasive methodologies. Their use and final validation are expected to contribute decisively to design clinical trials to test the efficacy of new drugs capable of halting diabetic retinal disease in the initial stages of the disease. Another fundamental step in this procedure is the characterization of the different phenotypes of diabetic retinal disease.

The design of future clinical trials should consider only groups of patients characterized by their homogeneity: patients presenting a specific retinopathy phenotype (wet/leaky or ischemic), with similar duration of diabetes and at similar levels of blood pressure and metabolic control (HbA_{1C} values).

4.3. Relevance for clinical management

It is accepted that in the initial stages of diabetic retinopathy when the fundus alterations detected by ophthalmoscopy or slit-lamp examination are limited to red-dot and hard or soft exudates, i.e., mild or nonproliferative DR, an annual examination is indicated to every patient with five or more years of duration of their diabetes.

This is the recommendation of the American Academy of Ophthalmology Guidelines for Diabetic Retinopathy (Fong and Ferris, 2003).

Our observations and the identification of different diabetic retinopathy phenotypes in the initial stages of DR, i.e., mild or moderate NPDR, characterized by different rates of progression of the retinopathy suggest that specific approaches should be used when managing these different retinopathy phenotypes.

A patient with mild or moderate NPDR, presenting retinopathy phenotype B (wet/leaky), characterized by marked breakdown of the BRB, identified by highly increased values of fluorescein leakage into the vitreous and a high red-dot formation rate, registered during a period of 1–2 years of follow-up, indicating fast retinopathy progression, should be watched more closely and examined at least at 6 months intervals. Furthermore, blood pressure values and metabolic control should be closely monitored at least at 3-month intervals and medication given to keep HbA_{1c} levels at $\leqslant 7.1\%$, systolic blood pressure at $\leqslant 140$ mmHg and diastolic blood pressure at $\leqslant 85$ mmHg. Communication channels should be rapidly established between ophthalmologist and their diabetologist, internist or general health care provider. Information should be given indicating that the chances of rapid retinopathy progression to more advanced stages of disease are in these patients relatively high, calling for immediate tighter control of both glycemia and blood pressure.

A patient with mild or moderate nonproliferative diabetic retinopathy presenting retinopathy phenotype C, ischemic, characterized by clear signs of capillary closure and variable red-dot formation rates would similarly indicate the need for shorter observation intervals than one year with particular attention for other systemic signs of microthrombosis. Here, however, control of hyperglycemia and blood pressure must be addressed with some degree of caution. Improved metabolic and blood pressure control must be progressive and less aggressive than with phenotype B. It is realized that the ischemia that characterizes phenotype C may become even more apparent in eyes submitted to rapid changes in metabolic control and lowering rapidly the blood pressure may increase the retinal damage associated with ischemia.

Finally, a patient with mild or moderate NPDR, presenting phenotype A, identified by low levels of fluorescein leakage, no signs of capillary closure, low red-dot formation rates and with a diabetes duration of more than 10 years, all signs indicating a slowly progression subtype of diabetic retinopathy may be followed at intervals longer than one year. If the examination performed at two years intervals confirms the initial phenotype characterization, the patient and his diabetologist, internist or general health care

provider should be informed of the good prognosis associated with this retinopathy phenotype.

4.4. Targeted treatments

It would be of great benefit to have a drug available which would prevent the need for photocoagulation and particularly one which may remove the other variables that remain a cause of concern. So many of these patients are not well controlled, they do not come to the doctor often, and they are going blind because they do not get medical attention in time for photocoagulation.

The major large clinical trials have shown that tight glycemic control slows the development and progression of diabetic retinopathy. But the constantly increasing incidence of type 2 diabetes and the evidence that retinal damage begins early on, underscore the need for a medical treatment that is targeted to the initial retinal alterations and to specific phenotypes of the retinal diabetic disease.

Several key pathways have been incriminated in the process of triggering diabetic retinal disease and they may play specific roles in the development of specific retinopathy phenotypes. Four candidates, the polyol pathway, nonenzymatic glucosylation, growth factors and protein-kinase C, may be playing leading roles in the development of diabetic retinal disease.

The polyol pathway theory holds that increased glucose metabolism, through the enzyme aldose–reductase interferes with sodium–potassium ATPase, damaging the retina (Greene et al., 1987).

The nonenzymatic glycosylation theory holds that the bonding of sugar molecules to other reactive molecules leads to critical retinal alterations and enhancement of processes of oxidative stress to the retina (Brownlee, 1994).

In the growth factor hypothesis, diabetes-induced damage promotes the liberation of growth factors that appear clearly as the best candidates to explain the developments of proliferative retinopathy. However, the potential role of growth factors in the initial stages and in nonproliferative retinopathy remains highly hypothetical (Clermont et al., 1997).

Finally, the protein kinase C theory. Many of the metabolic changes associated with hyperglycemia-induced oxidative stress, advanced glycosylation end-products of diacylglycerol through the polyol pathway, ultimately activate protein kinase C. In the retina, there is evidence that activation of the beta-isoform of PKC (PKC-beta) is associated with retinal vasodilatation and alterations in retinal blood flow, thus making PKC-beta an obvious target for intervention (Ishii et al., 1996).

A role for inflammation has also been proposed, and inflammation mediators have been suggested to be responsible for the increased fluorescein leakage observed in the initial stages of diabetes, by causing alterations in the tight junctions of the retinal vessels (Antonetti et al., 1998).

It is possible that all these different mechanisms of disease play complementary roles in the progression of diabetic retinal disease.

The identification of different retinopathy phenotypes characterized by different rates of progression and different dominant retinal alterations, may indicate that different disease processes predominate in specific retinopathy phenotypes, probably determined by specific gene mutations.

Individuals with a specific gene mutation which makes them more susceptible to the abnormal metabolic environment of diabetes will respond by developing a specific retinopathy phenotype.

Identification of well-defined retinopathy phenotypes appears to be an essential step in the quest for a successful treatment of diabetic retinopathy. After the characterization of specific retinopathy phenotypes, the predominant disease mechanisms involved may be identified and drugs directly targeted at the correction of these disease mechanisms used with greater chances of success.

Acknowledgments

This work was supported by grants from the Fundaç ão da Ciência e Tecnologia, Portugal and by the Alcon Institute Research Award. We thank present and former members of our research group as well as other collaborators for their participation in the work reported in this article. Among these we thank: José Rui Faria de Abreu, Maria Conceição Lobo, João Figueira, Isabel Pires, José Ludovico, Lilianne Duarte and Luísa Ribeiro.

References

Aiello, L.P., Gardner, T.W., King, G.L., Blankenship, G., Cavallerano, J.D., Ferris III, F.L., Kein, R., 1998. Diabetic retinopathy. Diabetes Care 21, 143–156.

Ang, A., Tong, L., Vernon, S.A., 2000. Improvement of reproducibility of macular volume measurements using the Heidelberg retinal tomograph. Br. J. Ophthalmol. 84, 1194–1197.

Antonetti, D.A., Barber, A.J., Khin, S., Lieth, E., Tarbell, J.M., Gardner, T.W., Penn State Retina Research Group, 1998. Vascular permeability in experimental diabetes is associated with reduced endothelial occluding content. Vascular endothelial growth factor decreases occludin in retinal endothelial cells. Diabetes 47, 1953–1959.

Ashton, N., 1963. Studies of retinal capillaries in relation to diabetic and others retinopathies. Br. J. Ophthalmol. 47, 521–538.

Ashton, N., 1965. The blood–retinal barrier and vasoglial relationships in retinal disease. Trans. Ophthalmol. Soc. UK 85, 199–230.

Ashton, N., 1974. Vascular basement membrane changes in diabetic retinopathy. Montgomery lecture, 1973. Br. J. Ophthalmol. 58, 344–347.

Bernardes, R., Lobo, C., Cunha-Vaz, J.G., 2002. Multimodal macula mapping: a new approach to study diseases of the macula. Surv. Ophthalmol. 47, 580–589.

Boeri, D., Maiello, M., Lorenzi, M., 2001. Increased prevalence of microthromboses in retinal capillaries of diabetic individuals. Diabetes 50, 1432–1439.

Brownlee, M., 1994. Glycation and diabetic complications (Lilly lecture 1993). Diabetes 43, 836–841.

Bursell, S.E., Clermont, A.C., Kinsley, B.T., Simonson, D.C., Aiello, L.M., Wolpert, H.A., 1996. Retinal blood flow changes in patients with insulin-dependent Diabetes mellitus and no diabetic retinopathy—a video fluorescein angiography study. Invest. Ophthalmol. 37, 886–897.

Cambien, F., Poirier, O., Lecerf, L., Evans, A., Cambou, J.-P., Arveiler, D., Luc, G., Bard, J.-M., Bara, L., Ricard, S., Tiret, L., Amouyel, P., Alhenc-Gelas, F., Soubrier, F., 1992. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. Nature 359, 641–644.

Carmo, A., Ramos, P., Reis, A., Proença, R., Cunha-Vaz, J.G., 1998. Breakdown of the inner and outer blood–retinal barriers in streptozotocin-induced diabetes. Exp. Eye. Res. 67, 569–575.

Clermont, A.C., Aiello, L.P., Mori, F., Aiello, L.M., Bursell, S.E., 1997. Vascular endothelial growth factor and severity of nonproliferative diabetic retinopathy mediate retinal hemodynamics in vivo: a potential role for vascular endothelial growth factor in the progression of nonproliferative diabetic retinopathy. Am. J. Ophthalmol. 124, 433–436.

Cogan, D.G., Kwabara, T., 1963. Capillary shunts in the pathogenesis of diabetic retinopathy. Diabetes 12, 293–300.

Cunha-Vaz, J.G., 1978. Pathophysiology of diabetic retinopathy. Br. J. Ophthalmol. 62, 351–355.

Cunha-Vaz, J.G., 1992. Perspectives in the treatment of diabetic retinopathy. Diabetes/Metabolism Rev. 8, 105–116.

Cunha-Vaz, J.G., 2000a. Blood–retinal barrier in diabetes. In: van Bijsterveld, P. (Ed.), Diabetic Retinopathy. Taylor & Francis, A Martin Dunitz, London, pp. 155–168.

Cunha-Vaz, J.G., 2000b. Diabetic Retinopathy. Surrogate Outcomes for drug development for diabetic retinopathy. Ophthalmologica 214, 377–380.

Cunha-Vaz, J.G., Travassos, A., 1984. Breakdown of the blood–retinal barriers and cystoid macular edema. Surv. Ophthalmol. 28, 485–492.

Cunha-Vaz, J.G., Faria de Abreu, J.R., Campos, A.J., Figo, G.M., 1975. Early breakdown of the blood–retinal barrier in diabetes. Br. J. Ophthalmol. 59, 649–656.

Cunha-Vaz, J.G., Fonseca, J.R., Faria de Abreu, J.F., 1978. Vitreous fluorophotometry and retinal blood flow studies in proliferative retinopathy. Albrecht Graefe's Arch. Klin. Exp. Ophthalmol. 207, 71–76.

Cunha-Vaz, J.G., Gray, J.R., Zeimer, R.C., Mota, M.C., Ishimoto, B.M., Leite, E., 1985a. Characterization of the early stages of diabetic retinopathy by vitreous fluorophotometry. Diabetes 34, 53–59.

Cunha-Vaz, J.G., Mota, C.C., Leite, E.C., Abreu, J.R., Ruas, M.A., 1985b. Effect of sulindac on the permeability of the blood–retinal barrier in early diabetic retinopathy. Arch. Ophthalmol. 103, 1307–1311.

Cunha-Vaz, J.G., Mota, C.C., Leite, E.C., Abreu, J.R., Ruas, M.A., 1986. Effect of sorbinil on blood–retinal barrier in early diabetic retinopathy. Diabetes 35, 574–578.

Cunha-Vaz, J.G., Lobo, C., Castro Sousa, J.P., Oliveiros, B., Leite, E., Faria de Abreu, J.R., 1998. Progression of retinopathy and alteration of the blood–retinal barrier in patients with type 2 diabetes: a seven-year prospective follow-up study. Graefe's Arch.Clin. Exp. Ophthalmol. 236, 264–268.

Cuypers, M.H.M., Kasanardjo, J.S., Polak, B.C.P., 2000. Retinal blood flow changes in diabetic retinopathy measured with the Heidelberg scanning laser Doppler flowmeter. Graefes Clin. Arch. Exp. Ophthalmol. 238, 935–941.

Darius, S., Wolf, G., Huang, P.L., Fishman, M.C., 1995. Localization of NADPH-diaphorase/nitric oxide synthase in the rat retina: an electron microscopic study. Brain Res. 690, 231–235.

Deng, D., Evans, T., Mukherjee, K., Downey, D., Chakrabarti, S., 1999. Diabetes-induced vascular dysfunction in the retina: role of endothelins. Diabetologia 42, 1228–1234.

Diabetes Control and Complications Trials Group, 2002. Effect of intensive therapy on the microvascular complications of type 1 diabetes mellitus. JAMA 287, 2563–2569.

Diabetic Retinopathy Study Research Group, 1981. Photocoagulation treatment of proliferative diabetic retinopathy: Clinical application of Diabetic Retinopathy Study (DRS) findings. Diabetic Retinopathy Study (DRS) Report Number 5. Dev. Ophthalmol. 2, 248–261.

Early Treatment Diabetic Retinopathy Study Research Group, 1991. Fundus photographic risk factors for progression of diabetic retinopathy. ETDRS Report Number 12. Ophthalmology 98, 823–833.

Engerman, R.L., 1976. Animal models of diabetic retinopathy. Trans. Am. Acad. Otolaryngol. 81, 710–715.

Engler, C., Krogsaa, B., Lund-Andersen, H., 1991. Blood–retina barrier permeability and its relation to the progression of diabetic retinopathy in type 1 diabetics. Graefe's Arch. Clin. Exp. Ophthalmol. 229, 442–446.

Ferris, F., Davis, M., 1990. Treating 20/20 eyes with diabetic macula edema. Arch. Ophthalmol. 117, 675–676.

Fong, D.S., Ferris, F., 2003. Practical management of diabetic retinopathy. Focal Points 21 (3), 1–17.

Fritsche, P., VanderHeijde, R., Suttorp-schulten, M.S.A., Pollack, B.C., 2002. Retinal thickness analysis (RTA). An objective method to assess and quantify the retinal thickness in healthy controls and diabetics without diabetic retinopathy. Retina 22, 768–771.

Gardner, T.W., Aiello, L.P., 2000. Pathogenesis of diabetic retinopathy. In: Flynn Jr., H.W., Smiddy, W.E. (Eds.), Diabetes and Ocular Disease:Past, Present, and Future Therapies, AAO Monograph No. 14. The Foundation of the American Academy of Ophthalmology, San Francisco, pp. 1–17.

Garner, A., 1987. Pathogenesis of diabetic retinopathy. Semin. Ophthalmol. 2, 4–11.

Ghirlanda, G., Di Leo, M.A.S., Caputo, S., Cercone, S., Greco, A.V., 1997. From functional to microvascular abnormalities in early diabetic retinopathy. Diabetes Metab. Rev. 13, 15–35.

Goldberg, M. F., Fine, S.L., (Eds.), 1969. Symposium on treatment of Diabetic Retinopathy. Washington, DC, US Department of Health, Education and Welfare.

Graier, W.F., Wascher, T.C., Lockner, L., Toplak, H., Krejs, G.J., Kukovetz, W.R., 1993. Exposure to elevated D-glucose concentrations modulates vascular endothelial cell vasodilatory responses. Diabetes 42, 1497–1505.

Grange, J.D., 1995. Retinopathie Diabétique. Rapport à la Société Française d'Ophthalmologie. Masson, Paris.

Greene, D.A., Lattimer, S.A., Sima, A.A.F., 1987. Sorbitol, phosphoinositides, and sodium–potassium-ATPase in the pathogenesis of diabetic complications. N. Engl. J. Med. 316, 599–606.

Hasegawa, T., Ravens, J.R., Toole, J.F., Salem, W., 1967. Precapillary arteriovenous anastomoses "thoroughfare channels" in the brain. Arch. Neurol. 16, 217–224.

Hee, M.R., Puliafito, C.A., Wong, C., Duker, J.S., Reichel, E., Rutledge, B., Schun, J.S., Swanson, E.A., Fujimoto, J.G., 1995. Quantitative assessment of macular edema with optical coherence tomography. Arch. Ophthalmol. 113 (8), 1019–1029.

Hee, M.R., Puliafito, C.A., Duker, J.C., Reichel, E., Coker, J.G., Wilkins, J.R., Schuman, J.S., Swanson, E.A., Fujimoto, J.G., 1998. Topography of diabetic macular edema with optical coherence tomography. Ophthalmology 105, 360–370.

Hellstedt, T., Immonen, I., 1996. Disappearance and formation rates of microaneurysms in early diabetic retinopathy. Br. J. Ophthalmol. 80, 135–139.

Holländer, H., Makarov, F., Dreher, Z., van Driel, D., Chan Ling, T., Stone, J., 1991. Structure of the macroglia of the retina: sharing and division of labour between astrocytes and Müeller cells. J. Comput. Neurol. 313, 587–603.

Hudson, H.L., Stickand, M.H., Futers, S., Grant, P.J., 2001. Effects of novel polymorphisms in the RAGE gene on transcriptional regulation and their association with diabetic retinopathy. Diabetes 50, 105:1511.

Humphries, S.E., Talmud, P.H., Montgomery, H., 2001. Gene–environment interaction: lipoprotein lipase and smoking and risk of CAD and the ACE and exercise-induced left ventricular hypertrophy as examples. In: Malcom, S., Gooship, J. (Eds.), Genotype to Phenotype. Bios Scientific, Oxford, pp. 55–72.

Ishii, H., Jirousek, M.R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S.E., Kern, T.S., Ballas, L.M., Heath, W.F., Stramm, L.E., Feener, E.P., King, G.L., 1996. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC ß inhibitor. Science 272, 728–731.

Kageman, L., Harris, A., Chung, H.S., Costa, V.P., Gargozi, H., 1999. Basics and limitations of color Doppler imaging. In: Pillmat, L.E., Harris, A., Anderson, D.R., Greve, E.L. (Eds.), Current Concepts on Ocular Blood Flow in Glaucoma. Kugler, The Hague, pp. 103–110.

Keavney, B., McKenzie, C., Parish, S., Palmer, A., Clark, S., Youngman, L., Delépine, M., Latharop, M., Peto, R., Collins, R., International Studies of Infarct Survial (ISIS) Collaborators, 2000. Large-scale test of hypothesised associations between the angiotensin-converting-enzyme insertion/deletion polymorphism and myocardial infarction in about 5000 cases and 6000 controls. Lancet 355, 434–442.

Keen, H., Chlouverakis, C., 1965. Metabolic factors in diabetic retinopathy. In: Graymore, C.N. (Ed.), Biochemistry of the Retina. Academic Press, New York, p. 123.

Klein, R., Klein, B.E.K., Moss, S.E., Cruikschanks, K.J., 1995a. The Wisconsin epidemiologic study of diabetic retinopathy. XV the long-term incidence of macular edema. Ophthalmology 102, 7–16.

Klein, R., Meuer, S.M., Moss, S.E., Klein, B.E.K., 1995b. Retinal microaneurysms counts and 10-year progression of diabetic retinopathy. Arch. Ophthalmol. 113, 1386–1391.

Klystra, J.A., Brown, J.C., Jaffe, G.J., Cox, T.A., Gallemore, R., Greven, C.M., Hall, J.G., Eifrig, D.E., 1999. The importance of fluorescein angiography in planning laser treatment of diabetic macular edema. Ophthalmology 106, 2068–2073.

Kohner, E.M., 1977. The problem of retinal blood flow in diabetes. In: Selected Topics in Diabetes, Proceedings of Int. Meeting Carlo Erba, S.p.A., Milan, Italy, pp. 15–24.

Kohner, E.M., Dollery, C.T., 1970. The rate formation and disappearance of microaneurysms in diabetic retinopathy. Eur. J. Clin. Invest. 1, 167–171.

Kohner, E.M., Hamilton, A.M., Saunders, S.J., Sutcliffe, B.A., Bulpitt, C.J., 1975. The retinal blood flow in diabetes. Diabetologica 27, 48–52.

Kohner, E.M., Sleightholm, M., The KROC collaborative study group., 1986. Does microaneurysm count reflect severity of early diabetic retinopathy? Ophthalmology 93, 586–589.

Leslie, R.D.G., Pyke, D.A., 1982. Diabetic retinopathy in identical twins. Diabetes 31, 19–21.

Lieth, E., Barber, A.J., Xu, B., Dice, C., Ratz, M.J., Tanase, D., Strother J.M., Penn State Retina Research Group, 1998. Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy. Diabetes 47, 815–820.

Lobo, C.L., Bernardes, R.C., Santos, F.J., Cunha-Vaz, J.G., 1999. Mapping retinal fluorescein leakage with confocal scanning laser fluorometry of the human vitreous. Arch. Ophthalmol. 117, 631–637.

Lobo, C.L., Bernardes, R.C., Cunha-Vaz, J.G., 2000. Alterations of the blood–retinal barriers and retinal thickness in preclinical retinopathy in subjects with type 2 diabetes. Arch. Ophthalmol. 118, 1664–1669.

Lobo, C.L., Bernardes, R.C., Figueira, J.P., Faria de Abreu, J.R., Cunha-Vaz, J.G., 2004. Three-year follow-up of blood–retinal barrier and retinal thickness alterations in patients with type 2 diabetes mellitus and mild nonproliferative diabetic retinopathy. Arch. Ophthalmol. 122, 211–217.

Lorenzi, M., Gerhardinger, C., 2001. Early cellular and molecular changes induced by diabetes in the retina. Diabetologia 44, 791–804.

Ludovico, J., Bernardes, R., Pires, I., Figueira, J., Lobo, C., Cunha-Vaz, J., 2003. Alterations of retinal capillary blood flow in preclinical retinopathy in subjects with type 2 diabetes. Graefe's Arch. Clin. Exp. Ophthalmol. 241, 181–186.

Malmfors, T., 1965. The adrenergic innervation of the eye as demonstrated by fluorescence microscopy. Acta. Physiol. Scand. 65, 259–266.

Matsumoto, A., Iwashima, Y., Abiko, A., Morikawa, A., Sekiguchi, M., Eto, M., Makino, I., 2000. Detection of the association between a deletion polymorphism in the gene encoding angiotensin I-converting enzyme and advanced diabetic retinopathy. Diabetes Res. Clin. Pract. 50, 195–202.

Michelson, G., Welzenbach, J., Pal, I., Harazny, J., 1998. Automatic full fields analysis of perfusion images gained by scanning laser Doppler flowmetry. Br. J. Ophthalmol. 82, 1294–1300.

Michelson, G., Welzenbach, J., Pal, I., Harazny, J., 2001. Functional imaging of the retinal microvasculature by scanning laser Doppler flowmetry. Int. Ophthalmol. 23, 327–333.

Murata, T., Ishibashi, T., Inomata, H., 1993. Immunohistochemical detection of blood–retinal barrier breakdown in streptozotocin-diabetic rats. Graefe's Arch. Clin. Exp. Ophthalmol. 231, 175–177.

Patel, A., Hibberd, M.L., Millward, B.A., Demaine, A.G., 1996. Chromosome 7q35 and susceptibility to diabetic micro-vascular complications. J. Diabetes Complications 10, 62–67.

Pires, I., Bernardes, R.C., Lobo, C.L., Soares, M.A., Cunha-Vaz, J.G., 2002. Retinal thickness in eyes with mild nonproliferative retinopathy in patients with type 2 diabetes mellitus Comparison of measurements obtained by retinal thickness analysis and optical coherence tomography. Arch. Ophthalmol. 120, 1301–1306.

Poitry-Yamate, C.L., Poitry, S., Tsacopoulos, M., 1965. Lactate released by Müller glial cells is metabolized by photoreceptors from mammalian retina. J. Neurosci. 15, 5179–5191.

Rema, M., Saravan, G., Deepa, R., Mohan, V., 2002. Familial clustering of diabetic in South Indian Type diabetic patients. Diabet. Med. 19 (11), 910–916.

Rigat, B., Hubert, C., Alhenc-Gelas, F., Cambien, F., Corvol, P., Soubrier, F., 1990. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. J. Clin. Invest. 86, 1343–1346.

Rungger-Brändle, E., Dosso, A.A., Leuenberger, P.M., 2000. Glial reactivity, an early feature of diabetic retinopathy. Invest. Ophthalmol. Vis. Sci. 41, 1971–1980.

Serrano-Rios, M., Regueiro, J.R., Serverino, R., Lopez-Larrea, C., Arnaiz-Villena, A., 1983. HLA antigens in insulin dependent and non-insulin dependent Spanish diabetic patients. Diabetes Metab. 9, 116–120.

Shahidi, M., Zeimer, R., Mori, M., 1990. Topography of retinal thickness in normals. Ophthalmology 97, 1120–1197.

Shahidi, M., Ogura, Y., Blair, N.P., Rusin, M.M., Zeimer, R., 1991. Retinal thickness analysis for quantitative assessment of diabetic macular edema. Arch. Ophthalmol. 109, 1115–1119.

Shahidi, M., Ogura, Y., Blair, N.P., Zeimer, R., 1994. Retinal thickness change after focal laser treatment of diabetic macular oedema. Br. J. Ophthalmol. 78 (11), 827–830.

Shakib, M., Cunha-Vaz, J.G., 1966. Studies on the permeability of the blood–retinal barrier. IV. Junctional complexes of the retinal vessels and their role in the permeability of the blood–retinal barrier. Exp. Eye Res. 5, 229–234.

Snieder, H., Sawtell, P.A., Ross, L., Walker, J., Spector, T.D., Leslie, R.D.G., 2001. HbA_{1C} levels are genetically determined even in type 1 diabetes. Evidence from healthy and diabetic twins. Diabetes 50, 2858–2863.

Stewart, L.L., Field, L.L., Ross, S., McArthur, R.G., 1993. Genetic risk factors in diabetic retinopathy. Diabetologia 36, 1293–1298.

Storm, C., Sander, B., Larsen, N., Larsen, M., Lund-Andersen, H., 2002. Diabetic macular edema assessed with optical coherence tomography and stereo fundus photography. Invest. Ophthalmol. Vis. Sci. 43, 241–245.

Stratton, I.M., Kohner, E.M., Aldington, S.J., Turner, R.C., Holman, R.R., Manley, S.E., Matthews, D.R., for the UKPDS Group, 2001. UKPDS 50: risk factors for incidence and progression of retinopathy in type II diabetes over 6 years from diagnosis. Diabetologia 44, 156–163.

Sullivan, P.M., Davies, G.E., Caldwell, G., Morris, A.C., Kohner, E.M., 1990. Retinal blood flow during hyperglycemia: a laser doppler velocimetry study. Invest. Ophthalmol. Vis. Sci. 31, 2041–2045.

Sutter, E.E., 2001. Imaging visual function with the multifocal m-sequence techniques. Vision Res. 41, 1241–1255.

Takagi, C., Bursell, S.E., Lin, Y.W., Takagi, H., Duh, E., Jiang, Z., Clermont, A.C., King, G.L., 1996. Regulation of retinal hemodynamics in diabetic rats by increased expression and action of endothelin-1. Invest. Ophthalmol. Vis. Sci. 37, 2504–2518.

Torrent-Solans, T., Duarte, L., Monteiro, R., Almeida, E., Bernardes, R., Cunha-Vaz, J., 2004. Red-dots counting on digitalized fundus images of mild nonproliferative retinopathy in Diabetes type 2. Invest. Ophthalmol. Vis. Sci. 2985 (Abstract number 2985/B620).

Tso, M.O.M., Cunha-Vaz, J.G., Shih, C.Y., Jones, C.W., 1980. Clinicopathologic study of blood–retinal barrier in experimental diabetes mellitus. Arch. Ophthalmol. 98, 2032–2040.

United Kingdom Prospective Diabetes Study, 1998. Diabetic retinopathy at diagnosis of type 2 diabetes and associated risk factors. Arch. Ophthalmol. 116, 297–303.

Van Schaik, H.J., Heintz, B., Larsen, M., Leite, E., Rosas, V., Schalnus, R., Van Best, J.A., 1997. Permeability of the blood–-retinal barrier in healthy humans. European concerted action on ocular fluorometry. Graefe's Arch. Clin. Exp. Ophthalmol. 235, 639–646.

Viñores, S.A., McGehee, R., Lee, A., Gadegbeku, C., Campochiaro, P.A., 1990. Ultrastructural localization of blood retinal–barrier breakdown in diabetic and galactosemic rats. J. Histochem. Cytochem. 38, 1341–1352.

Viñores, S.A., Van Niel, E., Swerdloff, J.L., Campochiaro, P.A., 1993. Electron microscopic immunocytochemical demonstration of blood–retinal barrier breakdown in human diabetics and its association with aldose reductase in retinal vascular endothelium and retinal pigment epithelium. Histochem. J. 25, 648–663.

Waltman, S.R., 1989. Sequential vitreous fluorophotometry in diabtes mellitus: a five year prospective study. Trans. Am. Ophthalmol. Soc. 82, 827–940.

Waltman, S.R., Krupin, T., Hanish, S., Oestrich, C., Becker, B., 1978a. Alteration of the blood–retinal barrier in experimental diabetes mellitus. Arch. Ophthalmol. 96, 878–879.

Waltman, S.R., Oestrich, C., Krupin, T., Hanish, S., Ratzan, S., Santiago, J., Kilo, C., 1978b. Quantitative vitreous fluorophotometry: a sensitive technique for measuring early breakdown of the blood–retinal barrier in young diabetic patients. Diabetes 27, 85–87.

Warpeha, K.M., Chakravarthy, U., 2003. Molecular genetics of microvascular disease in diabetic retinopathy. Eye 17, 305–311.

Warpeha, K.M., Ah-Fat, F., Harding, S., Patterson, C.C., Xu, W., Hart, P.M., Chakravarthy, U., Hughes, A.E., 1999. Dinucleotide repeat polymorphisms in EDN1 and NOS3 are not associated with severe diabetic retinopathy in type 1 or type 2 diabetes. Eye 13, 174–178.

Wasgenknecht, L.E., Bowden, D.W., Carr, J.J., Langefeld, C.D., Freedman, B.I., Rich, S.S., 2001. Familial aggregation of coronary artery calcium in families with type 2 diabetes. Diabetes 50, 861–866.

Wilkinson, C.P., Ferris III, F.L., Klein, R.E., Lee, P.P., Agardh, C.D., Davis, M., Dills, D., Kampik, A., Pararajasegaram, R., Verdaguer, J.T., representing the Global Diabetic Retinopathy Project Group, 2003. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology 101, 1677–1682.

Wise, G.N., Dollery, C.T., Henkid, P., 1971. The Retinal Circulation. Harper & Row, New York, pp. 421–454.

Zeimer, R., 1998. Application of retinal thickness analyzer to the diagnosis and management of ocular diseases. Ophthalmol. Clin. North America 11, 359–379.

Zeimer, R.C., Blair, N.P., Cunha-Vaz, J.G., 1983. Vitreous fluorophotometry for clinical research. I. Description and evaluation of a new fluorophotometer. Arch. Ophthalmol. 101, 1753–1756.